PATENT ABSTRACTS OF JAPAN

(11)Publication number : 2002-051780

(43)Date of publication of application : 19.02.2002

(51)Int.Cl. C12N 15/09
C12N 1/21
C12P 21/02
//(C12N 15/09
C12R 1:625)
(C12N 1/21
C12R 1:465)
(C12P 21/02
C12R 1:465)

(21)Application number : 2000-239132

(22)Date of filing : 07.08.2000

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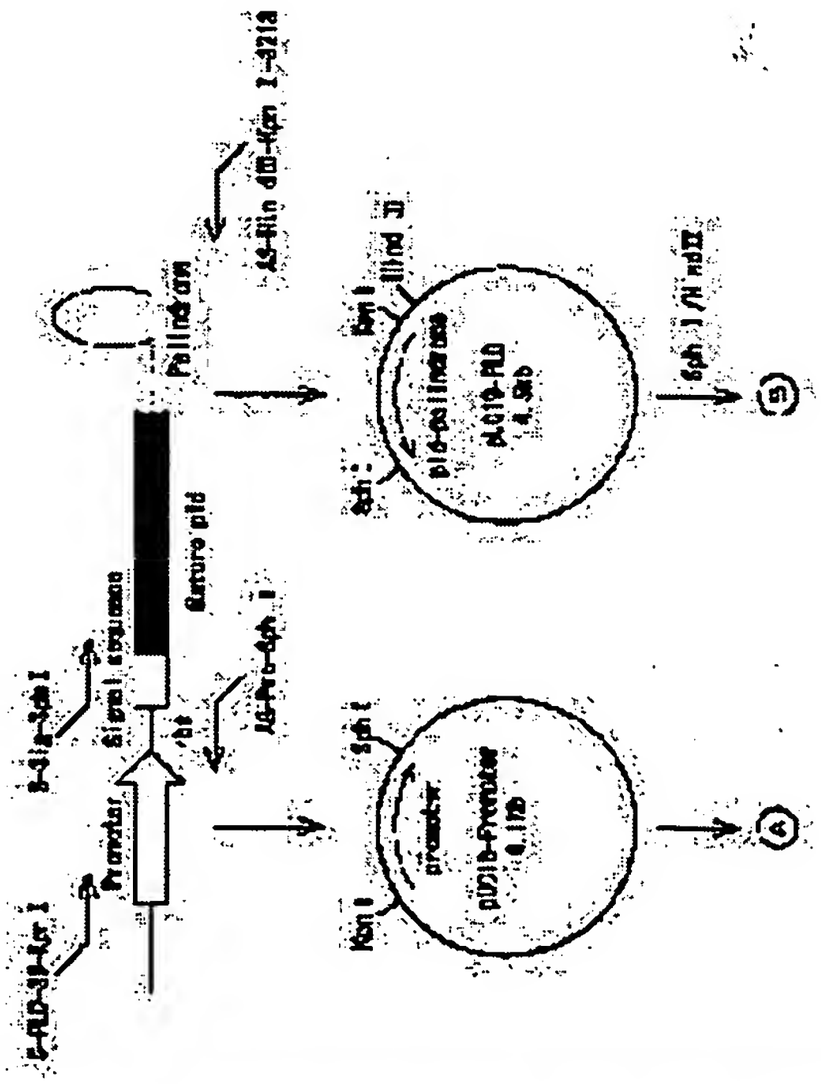
(54) PROMOTER, VECTOR HAVING THE SAME, RECOMBINANT MICROORGANISM, AND METHOD FOR PRODUCING PROTEIN

(57)Abstract:

PROBLEM TO BE SOLVED: To provide a promoter, which permits efficiently producing proteins such as phospholipase D, a vector using the same, a recombinant microorganism, and a method for producing the proteins.

SOLUTION: The first objective promoter is a base sequence derived from a DNA of Streptoverticillium cinnamoneum, is linked to the upstream side of a base sequence of protein for producing a protein, and has an activity of improving the ability of producing the above protein. The second objective vector is a vector using the promoter. The third objective recombinant microorganism and the fourth objective method for producing the protein are provided.



LEGAL STATUS

[Date of request for examination]

[Date of sending the examiner's decision of rejection]

[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] The promotor characterized by being the base sequence of the DNA origin of Streptoverticillium thinner MONIUMU, being combined with the upstream of the base sequence for protein for protein production, and having the improvement activity of the above-mentioned protein productivity.
[Claim 2] The promotor characterized by for 1 or some base sequences consisting of deletion and a base sequence permuted or added in the above-mentioned base sequence, being combined with the upstream of the base sequence for protein for protein production, and having the improvement activity of the above-mentioned protein productivity or it has a base sequence from the base number 38 in the array table of the array number 1 to the base number 1407.
[Claim 3] The vector characterized by having the base sequence for protein for protein production in the downstream of a promotor according to claim 1 or 2 and the above-mentioned promotor.
[Claim 4] The base sequence for protein is a vector according to claim 3 characterized by being an object for generation of HOSUFO lipase D.
[Claim 5] The base sequence for protein is a vector according to claim 4 characterized by being the thing of the DNA origin of Streptoverticillium thinner MONIUMU.
[Claim 6] The base sequence for protein is a vector according to claim 5 characterized by being the base sequence which 1 or some base sequences equipped with deletion and the protein productivity permuted or added in the base sequence or the above-mentioned base sequence from the base number 1512 in the array table of the array number 1 to the base number 3030.
[Claim 7] A vector given in claim 3 thru/or any of 6 they are. [which is characterized by having the palindromic sequence for suspending a translation of protein to the downstream of the base sequence for protein]
[Claim 8] A palindromic sequence is a vector according to claim 7 characterized by being the base sequence equipped with the function in which 1 or some base sequences suspend deletion and the translation of protein permuted or added in the base sequence or the above-mentioned base sequence from the base number 3031 in the array table of the array number 1 to the base number 3213.
[Claim 9] The recombination microorganism characterized by incorporating claim 3 thru/or which vector of 8 in a host.
[Claim 10] A host is a recombination microorganism according to claim 9 characterized by being an Actinomyces.
[Claim 11] A host is a recombination microorganism according to claim 10 characterized by being a streptomyces Ribi dance.
[Claim 12] The manufacture approach of the protein characterized by cultivating a recombination microorganism given in any [claim 9 thru/or] of 11 they are in culture medium, and producing the protein based on the base sequence for protein.

[Claim 13] The manufacture approach of the protein according to claim 12 characterized by adding a glucose to culture medium.

[Translation done.]

* NOTICES *

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the manufacture approach incorporating the promotor and the vector using it which can produce efficiently the protein of enzymes, such as HOSUFO lipase D (it is called PLD phospholipase D and the following), and the above-mentioned vector of protein rearrange and according to the above-mentioned recombination microorganism to a microorganism and a list.
[0002]
[Description of the Prior Art] Since PLD has high ester exchange reaction activity, the application to various kinds of useful phospholipid composition reactions is possible for it, and in order to extend the application range, cheap-ization by industrialization, i.e., mass production method, of PLD is expected.
[0003] From such usefulness of PLD, screening of the microorganism which carries out extensive secretory production of the PLD has been performed extensively. It is known as a result of the screening that only an Actinomyces group will carry out secretory production of a lot of PLD at present.
[0004] Also in these Actinomyceses group, as for Streptoverticillium thinner MONIUMU (IFO12852) (it is written as Stv.cinnamoneum (IFO12852) Streptoverticillium cinnamoneum and the following), it turns out in this time that it is the highest strain of secretion PLD activity (Nakajima et al., Biotechnol.Bioeng., Vol.44, and 1193-1198 (1994)).
[0005] The above-mentioned IFO number is a catalog number of the microorganism which can come to hand at a foundation fermentation lab (whereabouts, Osaka-shi) which is the domestic deposition engine of a microorganism. In addition, about Streptoverticillium, it may be classified into a streptomyces (Streptomyces) on a classification.
[0006] Moreover, although to change various medium compositions and culture conditions and to maximize the manifestation active mass of PLD about Stv.cinnamoneum of a wild strain conventionally using the culture medium which uses a meat extract as the base was tried, the manifestation activity was 2000 units / liter (it is hereafter written as U/L) extent at the maximum. With this specification, it is L in a unit. Unless it refuses especially, 1000 cm3 shall be shown.
[0007] Moreover, there is little twist contamination protein with easy purification of obtained PLD. By the case where a TSB culture medium is used, the manifestation active mass of PLD in Stv.cinnamoneum of a wild strain was still lower, and remained in 1500 U/L extent.
[0008]
[Problem(s) to be Solved by the Invention] However, PLD by which the content rate of PLD secreted in a culture medium was obtained from the still low thing took time in refining PLD from a culture medium, in the above-mentioned former, when the production cost of PLD became high, it became expensive, and the problem that the applicable range of PLD is restricted is produced.

[0009]
[Means for Solving the Problem] The promotor (promoter) of this invention is the base sequence of the DNA origin of Streptoverticillium thinner MONIUMU, in order to solve the above technical problem, and PLD etc. is a base sequence for protein for protein production (mat peptide). It is characterized by being combined with the upstream and having the improvement activity of the above-mentioned protein productivity.
[0010] Or it has a base sequence from the base number 38 in the array table of the array number 1 to the base number 1407 as the above-mentioned promotor, 1 or some base sequences consist of deletion and a base sequence permuted or added in the above-mentioned base sequence, it is combined with the upstream of the base sequence for protein for protein production, and you may have the improvement activity of the above-mentioned protein productivity.
[0011] According to the above-mentioned configuration, the productivity of protein, such as PLD, can be raised by having the above-mentioned base sequence of the DNA origin of Streptoverticillium thinner MONIUMU.
[0012] The vector of this invention is characterized by having the base sequence for protein for protein production in the downstream of the above-mentioned promotor and the above-mentioned promotor, in order to solve the above technical problem.
[0013] By the above-mentioned vector, the base sequence for protein may be an object for production of PLD, and may be the thing of the DNA origin of Streptoverticillium thinner MONIUMU.
[0014] Furthermore, in the base sequence or the above-mentioned base sequence from the base number [in / in the above-mentioned base sequence for protein / the array table of the array number 1] 1512 to the base number 3030, 1 or some base sequences may be deletion and a base sequence equipped with protein productivity permuted or added.
[0015] According to the above-mentioned configuration, it becomes possible by having the above-mentioned promotor to raise the protein productivity by the base sequences for protein for [of HOSUFO lipase D] generation of the DNA origin of for example, Streptoverticillium thinner MONIUMU etc.
[0016] In the above-mentioned vector, it is desirable to have the palindromic sequence (terminator) for suspending a translation of protein to the downstream of the base sequence for protein. According to the above-mentioned configuration, the productivity of protein, such as PLD, can be raised.
[0017] The recombination microorganism of this invention is characterized by incorporating which the above-mentioned vector in a host, in order to solve the aforementioned technical problem. By the above-mentioned recombination microorganism, a host may be an Actinomyces and may be a streptomyces Ribi dance.
[0018] By the incorporated promotor, it is improving, and the productivity of the protein which met the base sequence for protein included in the vector by the above-mentioned recombination microorganism is efficient, and can produce the above-mentioned protein in a culture medium at a high grade.
[0019] The manufacture approach of the protein of this invention is characterized by cultivating a recombination microorganism given in above any they are in culture medium, and producing the protein based on the base sequence for protein, in order to solve the aforementioned technical problem. It is desirable to add a glucose to culture medium by the above-mentioned manufacture approach.
[0020] According to the above-mentioned approach, by incorporating the above-mentioned promotor into the vector, it is efficient, and protein, such as PLD, can be secreted and produced in a high grade in a culture medium.
[0021]
[Embodiment of the Invention] It will be as follows if the gestalt of operation of this invention is explained based on drawing 1 thru/or drawing 10 .

[0022] (1) From an Actinomyces the DNA (for PLD generation) extract from the extract Actinomyces of DNA (genome) Treated the genetic manipulation technique about an Actinomyces. "Genetic Manupulation of Streptomyces" (Hopwood, D.A., Bibb, M.J., Chater, KF., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, and D.J. --) Smith, C.P., Ward, and J.M., and Schremph and H. (1985) "Genetic Manupulation of Streptomyces : it carried out according to a Laboratory Manual" and an approach given in the John InnesFoundation and Norwich.
[0023] The Actinomyces (Stv.cinnamoneum (IFO12852)) was sterilized. 100 mL The temperature of 30 degrees C performed shaking culture for 48 hours in the TORIPUTIKUSOI broth culture medium (it is called Tryptic Soy Broth, the following, and a TSB culture medium). The stainless steel coil of the magnitude whose diameters are about two to three cm was added into the culture medium at the time of this shaking culture. This controlled formation of the flocks-like fungus body under shaking culture.
[0024] Above TSB As a culture medium, they are pancreatic digest of casein 17.0 g/L, papaic digest of soybean meal 3.0 g/L, dextrose 2.5 g/L, sodium chloride 5.0 g/L, and dipotassium phosphate 2.5 g/L. Included Difco The shrine thing was used. In this specification, sterilization is annihilating the microorganism contained in solutions, such as a culture medium, by putting for 20 minutes by 121 ** under saturated steam.
[0025] The harvest of the obtained fungus body was carried out after culture termination, and it washed 3 times in TEbuffer (what sterilized 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA with the autoclave). In order to suspend a fungus body in TEbuffer of 5 mL after washing and to promote a bacteriolysis, they are a lysozyme and an AKUROMO peptidase, respectively 2 mg/mL and 4 mg/mL It was kept warm until the viscosity of a liquid became high at 30 degrees C in addition so that it might become (for about 30 minutes).
[0026] Then, they are pronase, EDTA, and SDS to a bacteriolysis solution. In addition, it was further kept warm at 37 degrees C for 2 hours. The chromosome DNA of an Actinomyces used with a phenol chloroform-extraction method and polyethylene glycerol settling was taken out from the bacteriolysis solution with the glass rod after incubation termination. At the end, it is RNase to the taken-out chromosome DNA. It processed and the extract operation of Chromosome DNA (genome) was ended. On these specifications, it is called a pld gene about the gene about generation of PLD.
[0027] Next, the decision of the DNA array of pld gene promoterregion and a valine DOROMU field is explained.
[0028] It is a restriction enzyme Sau three A1 about the chromosome DNA (genome) which the **** took out. It is a cloning vector pUC8 about the fragment DNA made to fragment after cutting, respectively. The genomic library was built by carrying out ligation and carrying out a transformation to Escherichia coli Nova Blue (product made from Novagen).
[0029] From the result of the amino acid sequence analysis of PLD, since about 25 amino acid sequences by the side of an amino acid end were checked, the DNA probe equivalent to the amino acid sequence was created, and the pld gene was identified by colony hybridization. Consequently, two kinds of fragments DNA which contain a pld gene partially were acquired.
[0030] One was the fragment DNA including the base sequence and its about 1500 upper bases (base) by the side of a PLD amino acid end, and another side was the fragment DNA including the base sequence, and its down-stream about 1000 bases (base) by the side of a PLD carboxyl group end. The base sequence of each fragment DNA was analyzed with the Sanger's method, and the map (base sequence) of a pld gene was built based on the overlapping base sequence.
[0031] From all the analyzed base sequences, the thing which are shown in an array table and which like and the palindromic sequence which has the function to make the downstream of a pld gene end a protein translation physically exist was checked.
[0032] Moreover, it describes above so that it may mention later. In the upstream of the pld gene from the high secretion manifestation active mass of PLD in the Actinomyces incorporating a pld gene, since it was assumed that a powerful manifestation promotor array existed, the field of the

base sequence of the upstream the about 1000 bases (base) which analysis ended was set up with promoterregion. The above-mentioned promoterregion is from the base number 38 in the array table of the array number 1 to the base number 1407.
[0033] (2) pUC702-promoter-pld For [which has the construction pld manifestation cassette of a plasmid] Actinomyceses It is an array table to [drawing 1 which prepared the pld secretion expression vector (pUC702-promoter-pld) in the following procedures thru/or drawing 3 , and a list Reference] a pld manifestation cassette -- the promoterregion (a promotor and ribosome joint site) of the upstream of a pld gene, and a pld gene (it consists of a signal sequence (sig peptide) and a mature pld gene (mat peptide)) -- and -- It has the palindromic sequence of the downstream of a pld gene.
[0034] The above-mentioned ribosome joint site is from the base number 1396 in the array table of the array number 1 to the base number 1403. The above-mentioned signal sequence is from the base number 1408 in the array table of the array number 1 to the base number 1509. The above-mentioned mature pld gene is a part corresponding to the PLD array after the secretion which does not include a signal sequence, and is from the base number 1510 in the array table of the array number 1 to the base number 3030. The above-mentioned palindromic sequence is from the base number 3031 in the array table of the array number 1 to the base number 3213.
[0035] Moreover, the amino acid number -1 shows a signal sequence from the amino acid number -34 in the array table of the array number 2. The amino acid number 505 shows the amino acid sequence of PLD from the amino acid number 1 in the array table of the array number 2.
[0036] Setting to construction of a plasmid, genetic manipulation is Molecular cloning fundamentally. It carried out according to law. Moreover, a restriction enzyme is New England Biolabs. The DNA ligation kit ver2 (TAKARA SHUZO make) was used for ligation for the shrine thing.
[0037] The procedure of plasmid construction is shown in drawing 1 thru/or drawing 3 . first, the Actinomyces Stv.cinnamoneum origin (IFO12852) shown in drawing 1 pld gene promoterregion and a pld gene -- and -- It consists of a palindromic sequence of the pld gene downstream. a pld manifestation cassette -- PCR (Polymerase Chain (amplification) Reaction) It amplified by law. Generally, since GC content is high, an Actinomyces is promoterregion. It halved to the pld gene-palindromic sequence and PCR magnification was performed.
[0038] The primer was designed so that TTG (leucine) which is the initiation codon which is in a signal sequence part then might be set to ATG (methionine). As PCR magnification conditions, it is 30 cycle ****** about this magnification as 15 seconds, 30 seconds, and 7 minutes at 94 degrees C, 60 degrees C, and 68 degrees C in the temperature and time amount of thermal denaturation, annealing, and an expanding reaction, respectively.
[0039] The chromosome of Stv.cinnamoneum was used as a template. In order to use KOD-plus-DNA-Polymerase (Toyobo make) and to gather the magnification effectiveness of PCR magnification, GC-Melt (Clonetech shrine make) was added to magnification at the system of reaction so that it might become 10% of final concentration.
[0040] As a primer, for magnification of promoterregion 5 '-GGGTACCACGTCATGGCGGGTCTCTCTCGTCCG-3' and (S-PLD-38-Kpn I) 5'-TCTGCATGCTGCATCCTTAAACGAAGTAACGATTCCGCG-3' (AS-Pro-Sph I) it is used -- for magnification of a pld gene-palindromic sequence 5 '-ACAGCATGCTCCGCCACCGGCTCCGCCGTTTACACCGCT-3' and (S-Sig-Sph I) 5'-GGTAAGCTTGGTACCATTTCCTCGCTGGTCGGTTCGGGGCCAGCGCAT-3' (AS-Hin dIII and Kpn I-3213) were used.
[0041] The fragmentation of the promoterregion amplified by the PCR method is Escherichia coli after carrying out ligation to pUC18 which cut with the restriction enzyme Kpn I and the restriction enzyme Sph I, and was cut with the same enzyme. A transformation is carried out to Nova Blue and it is plasmid pUC18-promoter. It obtained.
[0042] On the other hand, the fragmentation of a pld gene-palindromic sequence is restriction

enzymes Sph I and Hin dIII. It is Escherichia coli after carrying out ligation to pUC19 which cut to the site and was cut with the same enzyme. The transformation was carried out to Nova Blue and plasmid pUC19-pld was obtained.
[0043] Plasmid pUC18-promoter And the gene sequence of plasmid pUC19-pld was checked by the DNA sequencer (PE-biosystem gene analyzer 310).
[0044] Next, as shown in drawing 2 , they are a pUC19-pld plasmid to the restriction enzymes Sph I and Hin dIII. The fragmentation of a pld gene-palindromic sequence is started and, similarly they are restriction enzymes Sph I and Hin dIII. Cut pUC18-promoter It is Escherichia coli after carrying out ligation. A transformation is carried out to Nova Blue and it consists of a promoterregion-pld gene-palindromic sequence. Plasmid pUC18-promoter-pld which has a pld manifestation cassette It obtained.
[0045] On the other hand, as shown in drawing 3 , it is a shuttle vector pUC702. It prepared. pUC702 The vector pUC19 for Escherichia coli, and vector pIJ702 for Actinomyceses It is what was combined to Sac I and a Kpn I site, and prepared according to the approach of a publication to the following reports (report name: Molnar et al., J.Ferment.Bioeng., 72, and 368-372 (1991)).
[0046] Then, pUC18-promoter-pld From a plasmid The above-mentioned shuttle vector pUC702 which started the fragmentation containing a pld manifestation cassette with the restriction enzyme Kpn I, and was similarly cut with the restriction enzyme Kpn I It is Escherichia coli after carrying out ligation. A transformation is carried out to Nova Blue and it is plasmid pUC702-promoter-pld. It obtained. This plasmid pUC702-promoter-pld About whether the manifestation cassette was inserted in the right sense, it checked by the DNA sequencer.
[0047] (3) The Actinomyces (Stv.cinnamoneum) origin without promoterregion of the construction above of a pUC702-mature pld plasmid (IFO12852) Considering a pld gene expression vector as an example of a comparison, it is a shuttle vector pUC702. It used, and as shown in drawing 3 thru/or drawing 5 , it built.
[0048] First, the pld gene was made to amplify by two steps of PCR methods, as shown in drawing 4 . As conditions for First PCR, the temperature and time amount of thermal denaturation, annealing, and an expanding reaction are made into 45 seconds, 45 seconds, and 4 minutes at 98 degrees C, 52 degrees C, and 72 degrees C, respectively, and it is 25 cycle ****** about this magnification. The chromosome of Stv.cinnamoneum was used as a template. Using Pyrobest-DNA-polymerase (TAKARA SHUZO make), GC-Melt (Clonetech shrine make) was added to magnification at the system of reaction so that it might become 10% of final concentration.
[0049] As a primer for magnification, 5 '-CCCGGGAGCTGATAGCTTCTCCGCGTTGATCTTCC-3' (Genome-S) and 5'-CCATGATTACGAATTCCCGGGGATCTTGGT-3' (Genome2-AS) was used. The product after First PCR was used for the conditions of Second PCR as a template, and 5 '-TCGGAATTCGAGGTACCATGCTCCGCCACCGGCTCCGC-3' (Eco-Kpn-Sig-FW-PLD) and 5'-GCAGGTACCCCCCCTTGGCCGCGATTCCCG-3' (Kpn-Palind-RV-PLD) was used for them as a primer for magnification. The other magnification conditions are the same as that of First PCR.
[0050] Then, it is Escherichia coli after carrying out ligation to pUC19 which cut the amplified fragmentation (a signal sequence-mature pld gene-palindromic sequence is included) with the restriction enzyme Kpn I, and was cut with the same enzyme, as shown in drawing 5 after PCR. The transformation was carried out to Nova Blue and plasmid pUC19-pld was obtained. This plasmid The array of a pld gene was checked in the DNA sequencer.
[0051] pUC19-pld The plasmid was cut by Kpn I and the fragmentation (fragment DNA) containing a signal sequence-mature pld gene-palindromic sequence was collected. Shuttle vector pUC702 which was cut with the same enzyme and which is shown in drawing 3 It is Escherichia coli after carrying out ligation. By carrying out a transformation to Nova Blue, it is plasmid pUC702-pld. It obtained. This pUC702-pld Plasmid About whether the pld gene part was inserted in the right sense, it checked by the DNA sequencer.
[0052] (4) The transformation which the protoplast preparation Actinomyces protoplast of an

Actinomyces prepares and mentions later was performed according to the publication of "GeneticManupulation of Streptomyces" mentioned above. as an Actinomyces -- a streptomyces Ribi dance (1326 shares of Streptomyces lividans), the following, and S.lividans -- outlining -- it was used. S. 5 mL which sterilized lividans Inoculation was carried out to the TSB culture medium, and seed culture was performed for two - three days at 30 degrees C.
[0053] To then, a Sakaguchi flask 150 mL The YEME culture medium and the above-mentioned stainless steel coil were put in, inoculation of the seed culture liquid 1mL was carried out, and main culture was performed at 30 degrees C for 36 hours to 40 hours. The above-mentioned YEME culture medium is Difco yeast extract 3 g/L, Difco bacto-peptone 5 g/L, Difco malt extract 3 g/L, glucose 10 g/L, and sucrose 340 g/L. MgCl2.6H2O(2.5M) 2 mL/L after containing and sterilizing with an autoclave Addition and glycine(20%) 25 mL/L It contains.
[0054] The culture medium which contains the cultivated fungus body after culture termination (S. before the coloring matter of lividans becomes red) was taken out, and 3000 rpm and centrifugal actuation for 10 minutes were performed to the above-mentioned culture medium. It is 50 mL about the fungus body which remained except for supernatant liquid from the culture medium by which centrifugal was carried out. The sucrose solution washed twice 10.3%.
[0055] To the fungus body after washing, it is 15 mL. The fungus body suspension which added the lysozyme (lysozyme) solution (2 mg/mL inLbuffer, filter sterilization) was prepared until it became, and the above-mentioned fungus body suspension was kept warm for 30 minutes at 30 degrees C. The above-mentioned fungus body suspension was stirred well [whenever precipitate arises].
[0056] Then, it is 15 mL to the above-mentioned fungus body suspension. It filtered by the cotton wool yarn which added Pbuffer, and stirred well, then sterilized the above-mentioned fungus body suspension. 3000 rpm and centrifugal actuation for 7 minutes were performed for the filtrate after filtration.
[0057] Pbuffer was added to the fungus body obtained by removing supernatant liquid from the filtrate by which centrifugal was carried out. In measurement by the haemacytometer, the amount which adds Pbuffer was adjusted so that it might be set to four to 5x109/mL. The fungus body protoplast suspension solution prepared such was poured distributively in the sterilization tube, and was saved at -70 degrees C.
[0058] L (Lysis) buffer sucrose(10.3%) 100 mL, TES buffer(5.73 %, pH7.2)10 mL, K2SO4 (2.5 %) 1 mL and trace element solution 0.2 mL, KH2PO4 (0.5%) 1 mL and MgCl2.6H2O(2.5M) 0.1 mL, It is a lysozyme (product made from NAKARAI) just before use to the solution which added distilled water to CaCl2 (2.5M) 1 mL, set to 1 L, and carried out autoclave sterilization 2 mg/mL It adds so that it may become.
[0059] P(protoplast) buffer sucrose 10.3 g, K2SO4 0.025 g, MgCl2.6H2O 0.202 g, trace element solution 0.2 mL It melts with distilled water and is 80 mL. As opposed to what carried out and carried out autoclave sterilization KH2PO4 (0.5%) 1 mL [finishing / autoclave sterilization / apart from this respectively], CaCl2.2H2O(3.68 %) 10 mL and TES buffer(product made from NAKARAI, and TES what dissolved powder so that it might become 5.73%, and adjusted pH to 7.2)10 mL It adds, respectively.
[0060] trace element solution dissolves each in distilled water so that it may become ZnCl2 40 mg/L, FeCl3.6H2O 200 mg/L, CuCl2.2H2O 10 mg/L, MnCl2.4H2O 10 mg/L, Na2B4O7.10H2O 10 mg/L, and 6(NH4) Mo7O24.4H2O 10 mg/L.
[0061] (5) S.lividans which is the Actinomyces formed into the PUROTO pro strike as a transformation host of an Actinomyces as mentioned above was used. As mentioned above, the frozen object of the prepared suspension solution of the protoplast of S.lividans was thawed quickly, and 3000 rpm and centrifugal actuation for 7 minutes were performed at the room temperature. Then, plasmid solution given in (2) mentioned above 20 muL next, Tbuffer of 0.5 mL was added and pipetting was performed quickly.
[0062] Tbuffer was added, Pbuffer of 5 mL was added within 3 minutes, and centrifugal actuation

(for 3000 rpm and 30 seconds) was performed lightly. Except for supernatant liquid, to the residue which precipitated, Pbuffer of 0.5 mL was added, and it applied to homogeneity mostly on the front face of every [0.1 mL] and each R2YE plate, and was kept warm at 30 degrees C.
[0063] every applied one day - two days after -- a R2YE plate -- receiving -- Tiostreptone (the Wako Pure Chem make, 50 mg/mL in DMSO as Stock solution) Final concentration It added so that it might become 500microg/mL. 0.7% soft agar (product made from NAKARAI) 3 mL every -- it applied to homogeneity mostly and, similarly was kept warm at 30 degrees C, respectively. This completed the transformation (recombination microorganism) of Actinomyces S.lividans which is a host.
[0064] This recombination microorganism is deposited as name:Streptomyceslividans/pUC702-PLD (IFO 16465) to Institute for Fermentation, Osaka (Osaka). Moreover, Actinomyces S.lividans (recombination microorganism) which operated to the above-mentioned (3) similarly, and carried out the transformation to it also about the plasmid solution of a publication was obtained.
[0065] T(Transformation) buffer 75 mL SEPEG1000 which dissolved 35.8 g PEG1000 in distilled water Solution, sucrose(10.3%) 25 mL, the above-mentioned trace element solution0.2 mL, and K2SO4 (2.5 %) 1 mL are mixed. What carried out autoclave sterilization is taken 9.3 mL, and CaCl2 (5M) 0.2 mL [finishing / autoclave sterilization / separately] and Tris-maleic acid buffer 0.5 mL are added. Above-mentioned Tris-maleic acid buffer Tris buffer of 1 M It is a malic acid (maleic acid) about pH of a solution. 8.0 It adjusts.
[0066] An R2YE plate is an R2YE culture medium. 100 mL It receives and is 2.2 g. Autoclave sterilization of Difco Bacto agar is added and carried out. KH2PO4 (0.5%) 1 mL [finishing / autoclave sterilization / separately before the agar solidifies], CaCl2.2H2O(5M) 0.4 mL, L-proline (20%) 1.5mL, and NaOH(1 N) 0.7 mL are added, and specified quantity impregnation is carried out and it considers as an R2YE plate at a petri dish [finishing / sterilization]. An R2YE culture medium is sucrose 103 g, K2SO4 0.25 g, MgCl2.6H2O 10.12 g, glucose 10 g, Difco casaminoacids 0.1 g, trace element solution 2 mL, Difco yeast extract 5 g, and TES buffer 5.73 g. It dissolves in distilled water 1 L, and prepares.
[0067] (6) Recombination S.lividans obtained by the approach to have carried out culture **** of the recombination S.lividans (recombination microorganism) was cultivated using the aeration stirring mold culture apparatus. First, the above-mentioned Tiostreptone (final concentration 5microg/mL) was added. TSB culture medium 5 mL Inoculation was carried out to the put-in test tube from the R2YE plate, and it cultivated during the 2nd - the 3rd at 30 degrees C. To furthermore, a Sakaguchi flask TSB culture medium 100 mL Tiostreptone (final concentration g/mL of 5micro) is added, and it is the seed culture liquid of the above-mentioned test tube. 1 mL Inoculation was carried out and it cultivated during the 2nd - the 3rd at 30 degrees C further.
[0068] As main culture, it is a TSB culture medium to an aeration stirring mold culture apparatus (the Able, Inc. make, jar fermenter of 2 L (BMJ-02PI)). 1 L and Tiostreptone (final concentration 5microg/mL) are added, and it is the seed culture liquid of a Sakaguchi flask. Inoculation of the 50 mL was carried out. Culture is the culture temperature of 28 degrees C, pH=7.0, DO(dissolved oxygen concentration) = 2 ppm, and quantity of airflow. It carried out by setting it as 2 vvm and controlling.
[0069] (7) If the choline generated when the activity measurement PC of PLD (it is also called phosphatidylcholine and lecithin (Lecithin)) is made to hydrolyze by PLD is made to react to choline oxidase, a peroxidase, and a phenol, red quinone coloring matter will be generated. The activity of Above PLD was measured by measuring this coloring matter with a spectrophotometer using choline esterase B-Test Wako (Wako Pure Chem make). Suppose that the definition of the enzyme activity of one unit (U) is separated in the choline of 1micro mol (mol) in 1 minute.
[0070] First, a reaction substrate mixed solution is prepared. It is the mixed presentation per tube in the above-mentioned reaction substrate mixed solution Tris-HCl (pH=7.4)20 of H2O 40microL and 200 mM muL and 10mM CaCl2 10microL and 1%Triton X-100 10 muL and Lecithin Emulsion

10microL of 50 mg/mL It carried out. Above Lecithin Emulsion Lecithin 500 mg obtained from the egg 1 mL It dissolves in diethylether and is with water. 10 mL is made to carry out ultrasonic suspension of what carried out the scalpel rise.

[0071] By the activity measurement of PLD, it is a PLD enzyme solution. 10 muL It receives and is an above-mentioned reaction substrate mixed solution. 90 muL It mixed, stirred well and was made to react for 10 minutes at 37 degrees C. EDTA of 0.5 mM was added after the reaction and the reaction was stopped. Next, substrate enzyme agent 500 (choline oxidase, a peroxidase, phenol) muL In addition, it is kept warm for 5 minutes at 37 degrees C, and is the reaction stop solution 500. muL In addition, the reaction was stopped.

[0072] Next, a calibration curve is created. They are water and the criteria liquid of choline esterase B-Test Wako, respectively 150 muL 0 muL and 140 muL 10microL and 130 muL 20microL and 120 muL 30microL It mixed and those absorbances (wavelength 505 nm) were measured with the spectrophotometer, respectively. The calibration curve was created noting that the value of each absorbance was equivalent to the PLD activity used as 0 U/L, 500 U/L, 1000 U/L, and 1500 U/L. The PLD activity in a sample was determined using this calibration curve.

[0073] (8) Electrophoresis method (SDS-PAGE)

In order to check the secretion manifestation of PLD, SDS-PAGE electrophoresis was performed using 12.5% of separation gel. It sets to electrophoresis and is a culture-medium sample. 20 muL It used. Dyeing of gel was performed using coloring matter (Coomassie Brilliant Blue). The above-mentioned electrophoresis was performed in reference (Garfin et al., Methods Enzymol.182, and 425-441 (1990)) based on the approach of a publication.

[0074] (9) The purification of PLD by which secretory production was carried out into the purification culture medium of PLD is pH6. The cation exchange resin (Macroprep high S (Bio-Rad make etc.)) which can be set performed. Elution was performed by the stepwise elution (0.5 M) or the linear concentration gradient method (0-0.5 M) of a sodium chloride. Moreover, the effectiveness that heparin affinity chromatography was also equivalent was shown.

[0075] Artificers are Stv.cinnamoneum (IFO 12852). Origin Cloning of a pld gene was performed and the efficient production process using various gene expression systems of PLD rearrange and according to a microorganism was found out.

[0076] First, in before, although this invention persons tried construction of the manifestation system using Escherichia coli and yeast (Pichia pastoris) as a manifestation system of PLD using a recombination vector, as for the case of Escherichia coli, in the case of 1/20 and yeast, only one fourth of manifestation active masses were able to check to Stv.cinnamoneum of a wild strain with the highest manifestation activity ability. However, also by each above ****, since the volume of PLD as protein itself carried out the extensive check, since it was checked that PLD of the Actinomyces origin forms the right spacial configuration of a natural mold, it was considered that the manifestation active mass became low by these manifestation systems.

[0077] Although Yamane and others was also developing the secretory production system in Escherichia coli using PLD of the Actinomyces (Streptomyces antibioticus) origin, the manifestation active mass was about 3500 U/L extent at the maximum (Iwasaki et al., J.Ferment.Bioeng., 79, and 417-421 (1995)). Therefore, it was thought that development of the host-vector system of an Actinomyces was very important for development of the high secretory production system of Actinomyces PLD.

[0078] then, S.lividans whose this invention persons are kinds of an Actinomyces -- a host -- carrying out -- the Stv.cinnamoneum origin the manifestation system (shown in pUC702-pld, drawing 3 , or drawing 5) incorporating a pld gene -- further -- The manifestation system (shown in pUC702-promoter-pld, drawing 1 , or drawing 3) which incorporated promoterregion further was built to the pld gene. The transformation of the built class substitute plasmid was carried out to S.lividans which is an Actinomyces, respectively, and each S.lividans which carried out transformation was cultivated with the test tube.

[0079] Consequently, S.lividans/pUC702-pld In the manifestation system, although only the amount of manifestations equivalent to Stv.cinnamoneum of a wild strain was able to be checked, by the manifestation system (test tube culture 20000 U/L) of S.lividans/pUC702-promoter-pld, the remarkable big amount of manifestations (the amount of about 15 times) was able to be checked to Stv.cinnamoneum (test tube culture 1300 U/L) of a wild strain.
[0080] Therefore, the promotor concerning this invention is very powerful in the product from fungus body exogenous of protein, such as PLD for which it asks, and it was thought in the manifestation of various recombination protein including PLD that he was very effective.
[0081] Then, examination about optimization of the culture condition of this manifestation system S.lividans/pUC702-promoter-pld was performed using the aeration stirring mold cultivation tank. As conditions, to the above-mentioned TSB culture medium, it is making initiation addition glucose concentration into 0 g/l, 5 g/l, 15 g/l, and 30 g/l, and controlling it into the culture temperature of 28 degrees C, pH=7.0, and DO(dissolved oxygen concentration) = 2 ppm, respectively, and cultivated, respectively.
[0082] As shown in drawing 6 , when not adding a glucose, the activity of the secretion PLD in a culture medium is abbreviation. According to [although it was 20000 U/L as it is shown in drawing 7 thru/or drawing 9] the increment in the addition of a glucose, a PLD volume increases and is a glucose. 30 g/l By adding, it is abbreviation in a culture medium. It turns out that secretory production of the PLD activity of 30000 U/L can be carried out. This PLD activity is about 20 times the amount of manifestations of this compared with the PLD activity (the same TSB culture medium 1500 U/L) of Stv.cinnamoneum of a wild strain, and is about 15 times the amount of manifestations of this compared with the PLD activity (it optimizes, although it differs, and a culture medium is 2000 U/L by the case) of Stv.cinnamoneum of a wild strain.
[0083] However, it is initiation glucose concentration 30 g/l Even if it exceeded and having been enlarged, although cell mass increased, the volume of PLD did not increase. This was considered to be based on the effect of the catabolite repression resulting from existence of a superfluous glucose. The above-mentioned catabolite repression means that composition of many enzymes is checked with metabolite, such as catabolite repression, i.e., a glucose etc.
[0084] Furthermore, glucose concentration of initiation 15 g/l About the case, by electrophoresis (SDS-PAGE), the purity of PLD by which secretory production was carried out into the culture medium was checked, as shown in drawing 10 . In CBB coloring matter dyeing, contamination protein was hardly seen but PLD was able to check as a single band mostly.
[0085] It is abbreviation when the quantum of the PLD protein by which secretory production was carried out into this culture medium was carried out from the band of SDS-PAGE. It was 75 mg/L. It is abbreviation when the activity of PLD is calculated from this quantum value. It became 400 U/L. Before, this invention persons were the activity of the natural mold PLD which performed purification from Stv.cinnamoneum, and 468 U/L (Ogino et wl., J.Biochem., 125, and 263-269 (1999)). From this, PLD obtained in this invention had the value very near PLD of a natural mold.
[0086] Therefore, by the manufacture approach of PLD (protein) concerning this invention, PLD in a culture medium is high concentration, and it is a high grade, and moreover, since the activity of obtained PLD is also very close to PLD of a natural mold, it turns out that all of the trouble in various kinds of conventional manifestation systems including Escherichia coli were solvable.
[0087] Moreover, since the PLD purity by which the manufacture approach of PLD concerning this invention is secreted in a culture medium is high, after fungus body removal, with the ion exchange chromatography which used one step of ion exchange column, since separation recovery of PLD was possible, it could refine Above PLD very quickly and cheaply, and has mitigated the production cost of Above PLD.
[0088] as mentioned above, the PLD manifestation system of the Actinomyces (recombination microorganism) origin which included the promotor of the Actinomyces origin which this invention persons found out in the vector is very close to a natural mold -- high -- the fact that activity PLD

can be mass-produced with a high grade and a high concentration gestalt in a culture medium (that is, outside of a fungus body) shows that it is a very effective PLD manifestation system.
[0089] Thus, there are few manifestation systems which can carry out secretory production of the recombination protein to a high grade into a culture medium conventionally, and the application to various kinds of useful protein production other than Above PLD is assumed. That is, the manifestation cassette (promotor-signal sequence-palindromic sequence) of the vector (plasmid) concerning this invention was assumed to be a very effective thing in the secretory production of various useful protein including the secretory production of the useful protein of the various Actinomyces origins, such as cholesterol oxidase.
[0090] Moreover, S.lividans/pUC702-promoter-pld which is the recombination microorganism of this invention Like Stv.cinnamoneum (Fukuda et al., Biotechnol.Lett., 18, 951-956 (1996)) Since efficient immobilization to a microorganism maintenance particle (BSPs) can be performed, A mass production of useful protein including PLD using an immobilized cell is also possible, the productivity of useful protein can be raised by leaps and bounds, and cheap-ization of the above-mentioned useful protein can be attained.
[0091]
[Effect of the Invention] The promotor of this invention is the configuration of being the base sequence of the DNA origin of Streptoverticillium thinner MONIUMU, and it being combined with the upstream of the base sequence for protein for protein production, and having the improvement activity of the above-mentioned protein productivity as mentioned above.
[0092] The vector of this invention is the configuration of having the base sequence for protein for protein production, such as PLD, in the downstream of the above-mentioned promotor and the above-mentioned promotor, as mentioned above.
[0093] The recombination microorganism of this invention is the configuration that the above-mentioned vector is incorporated in hosts, such as an Actinomyces, as mentioned above.
[0094] The manufacture approach of the protein of this invention is an approach of cultivating the above-mentioned recombination microorganism in culture medium, and producing the protein based on the base sequence for protein as mentioned above.
[0095] So, by having used the above-mentioned promotor, the above-mentioned configuration and an approach can raise the productivity of protein, such as PLD, can carry out [cheap]-izing of the above-mentioned protein by industrialization, and do the effectiveness that applicability can be extended.
[0096]
[Layout Table]
<110> Foundation new industry creation research organization TLO hail -- Zaidanhoujin Shinsangyosouzoukenkyukikou TLO Hyogo <120> Promotor, The vector which has it, and a recombination microorganism, In a list, manufacture approach <160> 2 of protein <210> 1<211>3477<212> DNA <213> Streptoverticillium cinnamoneum <400> 1 tgcggggaga cccgggccgc ctgcaggccg gtctgtgacg tcatggcggg tctctctcgt 60 ccggtggcgg atgcgtcggt gtacggggct gccgtggccg gcacgtcagc gggccggggc 120 cgtcgggacg tccgtgagcc ccagctcggc ccgcgccgcg tcgaccatgtccctggcgtg 180 cagcagcccc agttcgccga gcgtgcgacg cagcgcctcc aggtcggcgg ccgtctccgc 240 cgccgcccgg cccagccggg cccgggcctt gaccaggccg agcgtggcca gcgcggcgcc 300 ccgcggctcg tccatcgccc ggaactcctc cagcgccccg ccgtaggtgg cccgtgcctc 360 ctcgaaccgg ccggcgcgga agaggacgtt gccgcgcatc ttatggttgt aggccagcgc 420 gctgaccagc cgcatccggc ggcaggtggc ctcggcctcg gtcagcagct ccagcgcgcg 480 ggcggtttcg ccccgcagcg agagcacgtc ggctatgccg cgcagggccc aggcgcggcc 540 gcgcgcgtcg tcggcgccac cggctatctc cgccgcctcc tcgaacatcg ccagggcggt 600 gtcgtaggag ccggtgttgc ggtgcatctg cgcgatgccc tccagcgccc acacggtgtg 660 ccgggcctcg ccccgccgtc tggcctcggc cagcagctgt tcgtgcagct cgccgacggc 720 ctggtagtcg cccctggatg cggccggtct cggccagacc ggccagggag tagccgcggg 780 ccacgacgtc cccgcgcgct cggccagccc ggcggccagg gcgagcaacc gccgggccag 840 ggcgagcgca ccgcgctgcc gggcgagggt gccgccgctc cacacggccc aggccatggc 900 gctctcgtca ccggcctgcc gtgccgcccg gtagctcgcc ttccacgcct

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Ser Ser Ala Ser Ala Thr Trp Ala Asp Gly His Pro Tyr Ala Leu 425 430 435 cac cac aag ctg gtg gcg gtc gac agc tcc gcc ttc tacatc ggc tcc 2874 His His Lys Leu Val Ala Val Asp Ser Ser Ala Phe Tyr Ile Gly Ser 440 445 450 aag aac ctc tac ccc tcg tgg ctg cag gac ttc ggc tac atc gtg gag 2922 Lys Asn Leu Tyr Pro Ser Trp Leu Gln Asp Phe Gly Tyr Ile Val Glu 455 460 465 470 agc ccg gag gcc gcc aag cag ctt gag gcc aag ctc ctc gac ccc gag 2970 Ser Pro Glu Ala Ala Lys Gln Leu Glu Ala Lys Leu Leu Asp Pro Glu 475 480 485 tgg aag ttc tcg cag gag acc gcg acg gtc gac cac gcg cgg ggc gtc 3018 Trp Lys Phe Ser Gln Glu Thr Ala Thr Val Asp His Ala Arg Gly Val 490 495 500 tgc tcg ctc tga gacgactgagcgcccggacg ttccgggggcg catgacgggg g ---- 3075 Cys Ser Leu 505 -- ccccccgt tcgctcatat aactacgttc gaatatcggg gaatgatccg atcgtcttca 3135 ctccatagtg aacggatttc attccgtgtc gctccggatg caaccatgcg ctggccccga 3195 accgaccagc gaggaaatct ggagattcaa tggagccatg tgggggaagt gcggtccgcc 3255 gctggggagc ggcgctgacc gcgtcggccg cggccatcgc ggtcgccctc ccgagcgcga 3315 acgccgaggc ggctgccggg ccgccgggaa tcgcggccaa gggggccttc ctgctcgaca 3375 gcgacgccaa ccgctccctg tggggcaagg cggccgacac ccggcggcag atggccagca 3435 ccaccaagat ccccgggaat tcgtaatcat ggtcatagct ga 3477 <210> 2 <211> 540<212> PRT<213> Streptoverticillium cinnamoneum <400> 2 Leu Leu Arg-His-Arg-Leu-Arg-Arg Leu His Arg Leu Thr-Arg-Ser-Ala -30 -25 -20 Ala Val Ser Ala Val Val Leu Ala Ala-Leu-Pro-Ala-Ala-Pro-Ala-Phe -15 -10 - 5 Ala Ser Ser Pro Ser Pro Ala Pro His Leu Asp Ala Val Glu Lys Ala - 1 1 5 10 Leu Arg Glu Val Ser Pro Gly Leu Glu Gly Asp Val Trp Gln Arg Thr 15 20 25 Asp Gly Asn Lys Leu Asp Ala Ser Ala Ala Asp Pro Ser Asp Trp Leu 30 35 40 45 LeuGln Thr Pro Gly Cys Trp Gly Asp Ala Ala Cys Lys Glu Arg Pro 50 55 60 Gly Thr Glu Arg Leu Leu Ala Lys Val Thr Glu Asn Ile Ser Lys Ala 65 70 75 Arg Arg Thr ValAsp Ile Ser Thr Leu Ala Pro PhePro Asn Gly Ala 80 85 90 Phe Gln Asp AlaIle Ala Ala Gly Leu Lys Ala Ser Val Ala Ser Gly 95 100 105 Asn Lys Pro Lys Val Arg Val Leu Val Gly Ala Ala Pro Val Tyr His 110 115 120 125 Met Asn Val Leu Pro Ser Lys Tyr Arg Asp Asp Leu Lys Ala Arg Leu 130 135 140 Gly Lys Ala AlaAspAsp Ile Thr Leu Asn Val Ala Ser Met Thr Thr 145 150 155 Ser Lys Thr Ser Phe Ser Trp Asn His Ser Lys Leu Leu Val Val Asp 160 165 170 Gly Glu Ser Ala Val Thr Gly Gly Ile Asn Ser Trp Lys Asp Asp Tyr 175 180 185 Val Asp Thr Gln His Pro Val Thr Asp Val Asp Leu Ala Leu Thr Gly 190 195 200 205 Pro Ala Ala SerSer Ala Gly Arg Tyr Leu Asp Thr Leu Trp Thr Trp 210 215 220 Thr Cys Gln Asn Lys Ser Asn IleAla Ser Val Trp Phe Ala Ala Ser 225 230 235 Gly Gly Asp Cys Met Ala Thr Met Glu Lys Asp Ala Asn Pro Arg Pro 240 245 250 Ala Gly Pro Thr Gly Asn Val Pro Val Ile Ala Val-Gly-Gly-Leu-Gly 255 260 265 Val Gly Ile Lys Asp Ser Asp Pro Ala-Ser-Thr-Phe-Arg-Pro Gln Leu 270 275 280 285 Pro Ser Ala Pro Asp Thr Lys Cys Val-Val-Gly-Leu-Pro-Asp Lys Thr 290 295300 Asn Ala Asp Arg Asp Tyr Asp Thr Val Asn Pro Glu Glu Ser Ala Leu 305 310 315 Arg Ala Leu Val Ala Ser Ala Asp Arg Gln Ile Val Ile Ser Gln Gln 320 325 330 Asp Leu Asn Ala Thr Cys Pro Pro Ile Ala Arg Tyr Asp Val Arg Leu 335 340 345 Tyr Asp Ile Leu Ala Ala Lys Met Ala Ala Gly Val Lys Val Arg Ile 350 355 360 365 Val Val Ser Asp Pro Ala Asn Arg Gly Ala Val Gly Ser Gly Gly Tyr 370 375 380 Ser Gln Ile Lys Ser Leu Ala Glu Ile Ser Asp Thr Leu Arg Asn Arg 385 390 395 Leu Ala Leu Leu Lys Gly Gly Asp Gln Gln Lys Ala Lys Ala Ala Met 400 405 410 Cys Ser Thr Leu Gln Leu Gly Thr Phe Arg Ser Ser Ala Ser Ala Thr 415 420 425 Trp Ala Asp Gly His Pro Tyr Ala Leu His His Lys Leu Val Ala Val 430 435 440 445 Asp Ser Ser Ala Phe Tyr Ile Gly Ser Lys Asn Leu Tyr Pro Ser Trp 450 455 460 Leu Gln Asp Phe Gly Tyr Ile Val Glu Ser Pro Glu Ala Ala Lys Gln 465 470 475 Leu Glu Ala Lys Leu Leu Asp Pro Glu Trp Lys Phe Ser Gln Glu Thr 480 485 490 Ala Thr Val Asp His Ala Arg Gly Val Cys Ser Leu 495 500 505

[Translation done.]

* NOTICES *

TECHNICAL FIELD

[Field of the Invention] This invention relates to the manufacture approach incorporating the promotor and the vector using it which can produce efficiently the protein of enzymes, such as HOSUFO lipase D (it is called PLD phospholipase D and the following), and the above-mentioned vector of protein rearrange and according to the above-mentioned recombination microorganism to a microorganism and a list.

[Translation done.]

* NOTICES *

PRIOR ART

[Description of the Prior Art] Since PLD has high ester exchange reaction activity, the application to various kinds of useful phospholipid composition reactions is possible for it, and in order to extend the application range, cheap-ization by industrialization, i.e., mass production method, of PLD is expected.

[0003] From such usefulness of PLD, screening of the microorganism which carries out extensive secretory production of the PLD has been performed extensively. It is known as a result of the screening that only an Actinomyces group will carry out secretory production of a lot of PLD at present.

[0004] Also in these Actinomyceses group, as for Streptoverticillium thinner MONIUMU (IFO12852) (it is written as Stv.cinnamoneum (IFO12852) Streptoverticillium cinnamoneum and the following), it turns out in this time that it is the highest strain of secretion PLD activity (Nakajima et al., Biotechnol.Bioeng., Vol.44, and 1193-1198 (1994)).

[0005] The above-mentioned IFO number is a catalog number of the microorganism which can come to hand at a foundation fermentation lab (whereabouts, Osaka-shi) which is the domestic deposition engine of a microorganism. In addition, about Streptoverticillium, it may be classified into a streptomyces (Streptomyces) on a classification.

[0006] Moreover, although to change various medium compositions and culture conditions and to maximize the manifestation active mass of PLD about Stv.cinnamoneum of a wild strain conventionally using the culture medium which uses a meat extract as the base was tried, the manifestation activity was 2000 units / liter (it is hereafter written as U/L) extent at the maximum. With this specification, it is L in a unit. Unless it refuses especially, 1000 cm3 shall be shown.

[0007] Moreover, there is little twist contamination protein with easy purification of obtained PLD. By the case where a TSB culture medium is used, the manifestation active mass of PLD in Stv.cinnamoneum of a wild strain was still lower, and remained in 1500 U/L extent.

[Translation done.]

* NOTICES *

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

EFFECT OF THE INVENTION

[Effect of the Invention] The promotor of this invention is the configuration of being the base sequence of the DNA origin of Streptoverticillium thinner MONIUMU, and it being combined with the upstream of the base sequence for protein for protein production, and having the improvement activity of the above-mentioned protein productivity as mentioned above.
[0092] The vector of this invention is the configuration of having the base sequence for protein for protein production, such as PLD, in the downstream of the above-mentioned promotor and the above-mentioned promotor, as mentioned above.
[0093] The recombination microorganism of this invention is the configuration that the above-mentioned vector is incorporated in hosts, such as an Actinomyces, as mentioned above.
[0094] The manufacture approach of the protein of this invention is an approach of cultivating the above-mentioned recombination microorganism in culture medium, and producing the protein based on the base sequence for protein as mentioned above.
[0095] So, by having used the above-mentioned promotor, the above-mentioned configuration and an approach can raise the productivity of protein, such as PLD, can carry out [cheap]-izing of the above-mentioned protein by industrialization, and do the effectiveness that applicability can be extended.
[0096]
[Layout Table]
<110> Foundation new industry creation research organization TLO hail -- Zaidanhoujin Shinsangyosouzoukenkyukikou TLO Hyogo <120> Promotor, The vector which has it, and a recombination microorganism, In a list, manufacture approach <160> 2 of protein <210> 1<211>3477<212> DNA <213> Streptoverticillium cinnamoneum <400> 1 tgcggggaga cccgggccgc ctgcaggccg gtctgtgacg tcatggcggg tctctctcgt 60 ccggtggcgg atgcgtcggt gtacggggct gccgtggccg gcacgtcagc gggccggggc 120 cgtcgggacg tccgtgagcc ccagctcggc ccgcgccgcg tcgaccatgtccctggcgtg 180 cagcagcccc agttcgccga gcgtgcgacg cagcgcctcc aggtcggcgg ccgtctccgc 240 cgccgcccgg cccagccggg cccgggcctt gaccaggccg agcgtggcca gcgcggcgcc 300 ccgcggctcg tccatcgccc ggaactcctc cagcgccccg ccgtaggtgg cccgtgcctc 360 ctcgaaccgg ccggcgcgga agaggacgtt gccgcgcatc ttatggttgt aggccagcgc 420 gctgaccagc cgcatccggc ggcaggtggc ctcggcctcg gtcagcagct ccagcgcgcg 480 ggcggtttcg ccccgcagcg agagcacgtc ggctatgccg cgcagggccc aggcgcggcc 540 gcgcgcgtcg tcggcgccac cggctatctc cgccgcctcc tcgaacatcg ccagggcggt 600 gtcgtaggag ccggtgttgc ggtgcatctg cgcgatgccc tccagcgccc acacggtgtg 660 ccgggcctcg ccccgccgtc tggcctcggc cagcagctgt tcgtgcagct cgccgacggc 720 ctggtagtcg cccctggatg cggccggtct cggccagacc ggccagggag tagccgcggg 780 ccacgacgtc cccgcgcgct cggccagccc ggcggccagg gcgagcaacc gccgggccag 840 ggcgagcgca ccgcgctgcc gggcgagggt gccgccgctc cacacggccc aggccatggc 900 gctctcgtca ccggcctgcc gtgccgcccg gtagctcgcc ttccacgcct gttcggcctc 960 ggcgacccgg cccagccgac ggctggcctc cgcgacggcg agcgccgcgt gcgccgactc 1020 cacgtccgtg ccgtgcagtt gggcccggcg ctgctgctcg acagccttgg ccagcacctc 1080 ctccagggag gagttgacgg acagctcgcc gagcgccccg cggtactcgg gggcgaatgc 1140 cttgccgtac atgtctgcct ctcttcgtga cggaccggcg

ggcgggaggc gggcgcgcgc 1200 cgcccgtccc gtgccgccct gccggtgatc acgaagctat ggcccggacc tatccgctga 1260 aatccctctc ggaggcggcc tgccgtacct ctgaggacgg gtggccgaag accgtgtcat 1320 ccgtgagatg tacggctccc gggagctgat agcttctccg cgttgatctt ccgttcgcgg 1380 aatcgttact tcgtttaagg atgcaca ttg ctc cgc cac cgg ctc cgc cgt tta 1434 Leu Leu Arg His Arg Leu Arg Arg Leu -30 cac-cgt-ctg-acc-cgc agt-gcg-gcg-gtc-tcg gcc gtc gtc ctg gcc gcc 1482 His Arg Leu Thr Arg Ser Ala-Ala-Val-Ser-Ala Val Val Leu Ala Ala -25 -20 -15 -10 ctg ccc gcg gct ccg gcc ttc gcg agc agc ccc tcg ccc gcc ccg cac 1530 Leu Pro Ala Ala Pro Ala Phe Ala Ser Ser Pro Ser Pro Ala Pro His - 5 - 1 1 5 ctg gac gcc gtg gag aag gcg ctg cgc gag gtc tca ccg ggg ctg gag 1578 Leu Asp Ala Val Glu Lys Ala Leu Arg Glu Val Ser Pro Gly Leu Glu 10 15 20 ggtgac gtctgg cag cgc acc gacggc aac aag ctg gac gcc tcc gcc 1626 Gly Asp Val Trp Gln Arg Thr Asp Gly Asn Lys Leu Asp Ala Ser Ala 25 30 35 gcggac ccc tcc gac tgg ctg ctg cag acc ccc ggt tgc tgg ggc gac 1674 AlaAsp Pro Ser Asp Trp Leu Leu Gln Thr Pro Gly Cys Trp Gly Asp 40 45 50 gcc gcg tgcaag gag cgt ccc ggc acc gag cgc ctg ctc gcc aag gtg 1722 Ala Ala Cys Lys Glu Arg Pro Gly Thr Glu Arg Leu Leu Ala Lys Val 55 60 65 70acg gagaac atc tcc aag gcc agg cgc acg gtg gac atc tcc acg ctc 1770 Thr Glu Asn Ile Ser Lys Ala Arg Arg Thr Val Asp Ile Ser Thr Leu 75 80 85 gcg ccc ttcccg aac ggt gcg ttc cag gac gcg ata gcc gcc ggc ctc 1818 Ala Pro Phe Pro Asn Gly Ala Phe Gln Asp Ala Ile Ala Ala Gly Leu 90 95 100 aag gcgtcg gtc gcg tcc ggc aac aag ccg aag gtc cgcgtc ctg gtc 1866 LysAla Ser Val Ala Ser Gly Asn Lys Pro Lys Val Arg Val Leu Val 105 110 115 ggcgcc gcg ccg gtc tac cac atg aac gta ctgccc tcg aag tac cgg 1914 Gly Ala Ala Pro Val Tyr His Met Asn Val Leu Pro Ser Lys Tyr Arg 120 125 130 gac gac ctc aag gcc cgg ctc ggc aag gcc gcc gac gac atc acg ctg 1962 Asp Asp Leu Lys A la Arg Leu Gly Lys Ala-Ala-Asp-Asp-Ile Thr Leu 135 140 145 150 aac gtc gcg tcg atg-acg-acg-tcg-aag acc agc ttc tcc tgg aac cac 2010 Asn Val Ala Ser Met Thr Thr Ser Lys Thr Ser Phe Ser Trp Asn His 155 160 165 tccaag ctc ctc gtc gtg gac ggc gag tcg gcc gtc acc ggt ggc atc 2058 Ser Lys Leu Leu Val Val Asp Gly Glu Ser Ala Val Thr Gly Gly Ile 170 175 180 aac agc tgg aag gac gac tac gtc gac acc cag cac ccg gtg acc gac 2106 Asn Ser Trp Lys Asp Asp Tyr Val Asp Thr Gln His Pro Val Thr Asp 185 190 195 gtggac ctg gcg ctg acc ggc ccc gcc gcg agc tcc gcc ggc cgc tac 2154 Val Asp Leu Ala Leu Thr Gly Pro Ala Ala Ser Ser Ala Gly Arg Tyr 200 205 210 ctg gac acg ctc tgg acg tgg acg tgc cag aac aag agcaac atc gcc2202 Leu Asp Thr Leu Trp Thr Trp Thr Cys Gln Asn Lys Ser Asn Ile Ala 215 220 225 230 agt gtg tgg ttc gcg gcc tcg ggc ggc gac tgc atg gcc acg atg gag 2250 Ser Val Trp Phe Ala Ala Ser Gly Gly Asp Cys Met Ala Thr Met Glu 235 240 245 aag gac gcg aac ccc agg ccc gcc ggg ccc acg ggc aac gtc ccc gtg 2298 Lys Asp Ala Asn Pro Arg Pro Ala Gly Pro Thr Gly Asn Val Pro Val 250 255 260 atc gcc gtg ggc ggc ctc ggc gtc ggc atc aag gac tcc gac ccc gcc 2346 Ile Ala Val Gly Gly Leu Gly Val Gly Ile Lys Asp Ser Asp Pro Ala 265 270 275 tcgacg ttc cgc ccg cag ctg ccc tcc gcc ccggac acc aag tgc gtc 2394 Ser Thr Phe Arg Pro Gln Leu Pro Ser Ala Pro Asp Thr Lys Cys Val 280 285 290 gtc ggc ctg ccc gac aag acc aac gcc gac cgt gac tac gac acg gtc2442 Val Gly Leu Pro Asp Lys Thr Asn Ala Asp Arg Asp Tyr Asp Thr Val 295 300 305 310aac ccc gag gag agc gcc ctg cgg gcc ctg gtg gcc agc gcc gac cgc 2490 Asn Pro Glu Glu Ser Ala Leu Arg Ala Leu Val Ala Ser Ala Asp Arg 315 320 325 cag-atc-gtc-atc-tcc cag-cag-gac-ctg-aac gcc acc tgc ccg ccc atc 2538 Gln Ile Val Ile Ser Gln Gln-Asp-Leu-Asn-Ala Thr Cys Pro Pro Ile 330 335 340 gcccgc tac gac gtc cgc ctc tac gac atc ctc gcc gcc aag atg gcg 2586 Ala Arg Tyr Asp Val Arg Leu Tyr Asp Ile Leu Ala Ala Lys Met Ala 345 350 355 gccggg gtg aag gtg cgc atc gtc gtc agc gac ccc gcc aac cgc ggc 2634 Ala Gly Val Lys Val Arg Ile Val Val Ser Asp Pro Ala Asn Arg Gly 360 365 370 gcg gtc ggc agc ggc ggc tac tcg cag atc aag tcc ctg gcc gag atc2682 Ala Val Gly Ser Gly Gly Tyr Ser Gln Ile Lys Ser Leu Ala Glu Ile 375 380 385 390 agc gac acg ctc cgc aac cgt ctc gcc ctg ctc aag ggc ggc gac cag 2730 Ser Asp Thr Leu Arg Asn Arg Leu Ala Leu Leu Lys Gly Gly Asp Gln 395 400 405 cag aag gcc aag gcg gcc atg tgc tcc acc ctc cag ctg ggg acc ttc 2778 Gln Lys Ala Lys Ala Ala Met Cys Ser Thr Leu Gln Leu Gly Thr Phe 410 415 420 cgc agc tcc gcg agc gcc acg tgg gcc gac ggg cac ccc tac gcc ctg 2826 Arg Ser Ser Ala Ser Ala Thr Trp Ala Asp Gly His Pro Tyr Ala Leu 425 430 435 caccac aag ctg gtg gcg gtc gac agc tcc gccttc tac atc ggc tcc 2874 His His Lys Leu Val Ala Val Asp Ser Ser Ala Phe Tyr Ile Gly Ser 440 445 450 aag aac ctc tac ccc tcg tgg ctg cag gac ttc ggc tac atc gtg gag2922 Lys Asn Leu Tyr Pro

Ser Trp Leu Gln Asp Phe Gly Tyr Ile Val Glu 455 460 465 470agc ccg gag gcc gcc aag cag ctt gag gcc aag ctc ctc gac ccc gag 2970 Ser Pro Glu Ala Ala Lys Gln Leu Glu Ala Lys Leu Leu Asp Pro Glu 475 480 485 tgg aag ttc tcg cag gag acc gcg acg gtc gac cac gcg cgg ggc gtc 3018 Trp Lys Phe Ser Gln Glu Thr Ala Thr Val Asp-His-Ala-Arg-Gly Val 490 495 500 tgc-tcg-ctc-tga-gacgactgag cgcccggacg ttccggggcg-catgacgggg g---- 3075 Cys Ser-Leu 505 --ccccccgt-tcgctcatat aactacgttc-gaatatcggg gaatgatccg-atcgtcttca 3135 ctccatagtg aacggatttc attccgtgtc gctccggatg caaccatgcg ctggccccga3195 accgaccagc gaggaaatct ggagattcaa tggagccatg tgggggaagt gcggtccgcc 3255 gctggggagc ggcgctgacc gcgtcggccg cggccatcgc ggtcgccctc ccgagcgcga 3315 acgccgaggc ggctgccggg ccgccgggaa tcgcggccaa gggggccttc ctgctcgaca 3375 gcgacgccaa ccgctccctg tggggcaagg cggccgacac ccggcggcag atggccagca 3435 ccaccaagat ccccgggaat tcgtaatcat ggtcatagct ga 3477 <210> 2<211> 540 <212> PRT <213> Streptoverticillium cinnamoneum <400> 2 Leu Leu Arg HisArg Leu Arg Arg Leu His Arg Leu Thr Arg Ser Ala -30 - 25 - 20 Ala Val Ser Ala Val Val Leu Ala Ala Leu Pro Ala Ala Pro Ala Phe - 15 -10 - 5 Ala Ser Ser Pro Ser Pro Ala Pro His Leu Asp Ala Val Glu Lys Ala - 1 1 5 10 Leu Arg Glu Val Ser Pro Gly Leu Glu Gly Asp Val Trp Gln Arg Thr 15 20 25 Asp Gly Asn Lys Leu Asp Ala Ser Ala Ala Asp Pro Ser Asp Trp Leu 30 35 40 45 LeuGln Thr Pro Gly Cys Trp Gly Asp Ala Ala Cys Lys Glu Arg Pro 50 55 60 Gly Thr Glu Arg Leu Leu Ala Lys Val Thr Glu Asn Ile Ser Lys Ala 65 70 75 Arg Arg Thr ValAsp Ile Ser Thr LeuAla Pro Phe Pro Asn Gly Ala 80 85 90 Phe Gln Asp AlaIle Ala Ala Gly Leu Lys Ala Ser Val Ala Ser Gly 95 100 105 Asn Lys Pro Lys Val Arg Val Leu Val Gly Ala Ala Pro Val Tyr His 110 115 120 125 Met Asn Val Leu Pro Ser Lys Tyr Arg Asp Asp Leu Lys Ala Arg Leu 130 135 140 Gly Lys Ala AlaAspAsp Ile Thr Leu Asn Val Ala Ser Met Thr Thr 145 150155 Ser Lys Thr Ser Phe Ser Trp Asn His SerLys Leu Leu-Val-Val-Asp 160 165 170 Gly Glu Ser Ala Val Thr Gly Gly Ile-Asn-Ser-Trp-Lys-Asp-Asp-Tyr 175 180 185 Val Asp Thr Gln His Pro Val Thr Asp Val Asp Leu Ala Leu Thr Gly 190 195 200 205 Pro Ala Ala Ser Ser Ala Gly Arg Tyr Leu Asp Thr Leu Trp Thr Trp 210 215 220 Thr Cys Gln Asn Lys Ser Asn Ile Ala Ser Val Trp Phe Ala Ala Ser 225 230 235 Gly Gly Asp Cys Met Ala Thr Met Glu Lys Asp Ala Asn Pro Arg Pro 240 245 250 Ala Gly Pro Thr Gly Asn Val Pro Val Ile Ala Val Gly Gly Leu Gly 255 260 265 Val Gly Ile Lys Asp Ser Asp Pro Ala Ser Thr Phe Arg Pro Gln Leu 270 275 280 285 Pro Ser Ala Pro Asp Thr Lys Cys Val Val Gly Leu Pro Asp Lys Thr 290 295 300 Asn Ala Asp Arg Asp Tyr Asp Thr Val Asn Pro Glu Glu Ser Ala Leu 305 310 315 Arg Ala Leu Val Ala Ser Ala Asp Arg Gln Ile Val Ile Ser Gln Gln 320 325 330 Asp Leu Asn Ala Thr Cys Pro Pro Ile Ala Arg Tyr Asp Val Arg Leu 335 340 345 Tyr Asp Ile Leu Ala Ala Lys Met Ala Ala Gly Val Lys Val Arg Ile 350 355 360 365 Val Val Ser Asp Pro Ala Asn Arg Gly Ala Val Gly Ser Gly Gly Tyr 370 375 380 Ser Gln Ile Lys Ser Leu Ala Glu Ile Ser Asp Thr Leu Arg Asn Arg 385 390 395 Leu Ala Leu Leu Lys Gly Gly Asp Gln Gln Lys Ala Lys Ala Ala Met 400 405 410 Cys Ser Thr Leu Gln Leu Gly Thr Phe Arg Ser Ser Ala Ser Ala Thr 415 420 425 Trp Ala Asp Gly His Pro Tyr Ala Leu His His Lys Leu Val Ala Val 430 435 440 445 Asp Ser Ser Ala Phe Tyr Ile Gly Ser Lys Asn Leu Tyr Pro Ser Trp 450 455 460 Leu Gln Asp Phe Gly Tyr Ile Val Glu Ser Pro Glu Ala Ala Lys Gln 465 470 475 Leu Glu Ala Lys Leu Leu Asp Pro Glu Trp Lys Phe Ser Gln Glu Thr 480 485 490 Ala Thr Val Asp His Ala Arg Gly Val Cys Ser Leu 495 500 505

[Translation done.]

* NOTICES *

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] However, PLD by which the content rate of PLD secreted in a culture medium was obtained from the still low thing took time in refining PLD from a culture medium, in the above-mentioned former, when the production cost of PLD became high, it became expensive, and the problem that the applicable range of PLD is restricted is produced.

[Translation done.]

* NOTICES *

MEANS

[Means for Solving the Problem] The promotor (promoter) of this invention is the base sequence of the DNA origin of Streptoverticillium thinner MONIUMU, in order to solve the above technical problem, and PLD etc. is a base sequence for protein for protein production (mat peptide). It is characterized by being combined with the upstream and having the improvement activity of the above-mentioned protein productivity.
[0010] Or it has a base sequence from the base number 38 in the array table of the array number 1 to the base number 1407 as the above-mentioned promotor, 1 or some base sequences consist of deletion and a base sequence permuted or added in the above-mentioned base sequence, it is combined with the upstream of the base sequence for protein for protein production, and you may have the improvement activity of the above-mentioned protein productivity.
[0011] According to the above-mentioned configuration, the productivity of protein, such as PLD, can be raised by having the above-mentioned base sequence of the DNA origin of Streptoverticillium thinner MONIUMU.
[0012] The vector of this invention is characterized by having the base sequence for protein for protein production in the downstream of the above-mentioned promotor and the above-mentioned promotor, in order to solve the above technical problem.
[0013] By the above-mentioned vector, the base sequence for protein may be an object for production of PLD, and may be the thing of the DNA origin of Streptoverticillium thinner MONIUMU.
[0014] Furthermore, in the base sequence or the above-mentioned base sequence from the base number [in / in the above-mentioned base sequence for protein / the array table of the array number 1] 1512 to the base number 3030, 1 or some base sequences may be deletion and a base sequence equipped with protein productivity permuted or added.
[0015] According to the above-mentioned configuration, it becomes possible by having the above-mentioned promotor to raise the protein productivity by the base sequences for protein for [of HOSUFO lipase D] generation of the DNA origin of for example, Streptoverticillium thinner MONIUMU etc.
[0016] In the above-mentioned vector, it is desirable to have the palindromic sequence (terminator) for suspending a translation of protein to the downstream of the base sequence for protein. According to the above-mentioned configuration, the productivity of protein, such as PLD, can be raised.
[0017] The recombination microorganism of this invention is characterized by incorporating which the above-mentioned vector in a host, in order to solve the aforementioned technical problem. By the above-mentioned recombination microorganism, a host may be an Actinomyces and may be a streptomyces Ribi dance.
[0018] By the incorporated promotor, it is improving, and the productivity of the protein which met the base sequence for protein included in the vector by the above-mentioned recombination microorganism is efficient, and can produce the above-mentioned protein in a culture medium at a

high grade.
[0019] The manufacture approach of the protein of this invention is characterized by cultivating a recombination microorganism given in above any they are in culture medium, and producing the protein based on the base sequence for protein, in order to solve the aforementioned technical problem. It is desirable to add a glucose to culture medium by the above-mentioned manufacture approach.
[0020] According to the above-mentioned approach, by incorporating the above-mentioned promotor into the vector, it is efficient, and protein, such as PLD, can be secreted and produced in a high grade in a culture medium.
[0021]
[Embodiment of the Invention] It will be as follows if the gestalt of operation of this invention is explained based on drawing 1 thru/or drawing 10 .
[0022] (1) From an Actinomyces the DNA (for PLD generation) extract from the extract Actinomyces of DNA (genome) Treated the genetic manipulation technique about an Actinomyces. "Genetic Manupulation of Streptomyces" (Hopwood, D.A., Bibb, M.J., Chater, KF., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, and D.J. --) Smith, C.P., Ward, and J.M., and Schremph and H. (1985). "Genetic Manupulation of Streptomyces : it carried out according to a Laboratory Manual" and an approach given in the John InnesFoundation and Norwich.
[0023] The Actinomyces (Stv.cinnamoneum (IFO12852)) was sterilized. 100 mL The temperature of 30 degrees C performed shaking culture for 48 hours in the TORIPUTIKUSOI broth culture medium (it is called Tryptic Soy Broth, the following, and a TSB culture medium). The stainless steel coil of the magnitude whose diameters are about two to three cm was added into the culture medium at the time of this shaking culture. This controlled formation of the flocks-like fungus body under shaking culture.
[0024] Above TSB As a culture medium, they are pancreatic digest of casein 17.0 g/L, papaic digest of soybean meal 3.0 g/L, dextrose 2.5 g/L, sodium chloride 5.0 g/L, and dipotassium phosphate 2.5 g/L. Included Difco The shrine thing was used. In this specification, sterilization is annihilating the microorganism contained in solutions, such as a culture medium, by putting for 20 minutes by 121 ** under saturated steam.
[0025] The harvest of the obtained fungus body was carried out after culture termination, and it washed 3 times in TEbuffer (what sterilized 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA with the autoclave). In order to suspend a fungus body in TEbuffer of 5 mL after washing and to promote a bacteriolysis, they are a lysozyme and an AKUROMO peptidase, respectively 2 mg/mL and 4 mg/mL It was kept warm until the viscosity of a liquid became high at 30 degrees C in addition so that it might become (for about 30 minutes).
[0026] Then, they are pronase, EDTA, and SDS to a bacteriolysis solution. In addition, it was further kept warm at 37 degrees C for 2 hours. The chromosome DNA of an Actinomyces used with a phenol chloroform-extraction method and polyethylene glycerol settling was taken out from the bacteriolysis solution with the glass rod after incubation termination. At the end, it is RNase to the taken-out chromosome DNA. It processed and the extract operation of Chromosome DNA (genome) was ended. On these specifications, it is called a pld gene about the gene about generation of PLD.
[0027] Next, the decision of the DNA array of pld gene promoterregion and a valine DOROMU field is explained.
[0028] It is a restriction enzyme Sau three A1 about the chromosome DNA (genome) which the **** took out. It is a cloning vector pUC8 about the fragment DNA made to fragment after cutting, respectively. The genomic library was built by carrying out ligation and carrying out a transformation to Escherichia coli Nova Blue (product made from Novagen).
[0029] From the result of the amino acid sequence analysis of PLD, since about 25 amino acid sequences by the side of an amino acid end were checked, the DNA probe equivalent to the amino acid sequence was created, and the pld gene was identified by colony hybridization. Consequently,

two kinds of fragments DNA which contain a pld gene partially were acquired.
[0030] One was the fragment DNA including the base sequence and its about 1500 upper bases (base) by the side of a PLD amino acid end, and another side was the fragment DNA including the base sequence, and its down-stream about 1000 bases (base) by the side of a PLD carboxyl group end. The base sequence of each fragment DNA was analyzed with the Sanger's method, and the map (base sequence) of a pld gene was built based on the overlapping base sequence.
[0031] From all the analyzed base sequences, the thing which are shown in an array table and which like and the palindromic sequence which has the function to make the downstream of a pld gene end a protein translation physically exist was checked.
[0032] Moreover, it describes above so that it may mention later. In the upstream of the pld gene from the high secretion manifestation active mass of PLD in the Actinomyces incorporating a pld gene, since it was assumed that a powerful manifestation promotor array existed, the field of the base sequence of the upstream the about 1000 bases (base) which analysis ended was set up with promoterregion. The above-mentioned promoterregion is from the base number 38 in the array table of the array number 1 to the base number 1407.
[0033] (2) pUC702-promoter-pld For [which has the construction pld manifestation cassette of a plasmid] Actinomyceses It is an array table to [drawing 1 which prepared the pld secretion expression vector (pUC702-promoter-pld) in the following procedures thru/or drawing 3 , and a list Reference] a pld manifestation cassette -- the promoterregion (a promotor and ribosome joint site) of the upstream of a pld gene, and a pld gene (it consists of a signal sequence (sig peptide) and a mature pld gene (mat peptide)) -- and -- It has the palindromic sequence of the downstream of a pld gene.
[0034] The above-mentioned ribosome joint site is from the base number 1396 in the array table of the array number 1 to the base number 1403. The above-mentioned signal sequence is from the base number 1408 in the array table of the array number 1 to the base number 1509. The above-mentioned mature pld gene is a part corresponding to the PLD array after the secretion which does not include a signal sequence, and is from the base number 1510 in the array table of the array number 1 to the base number 3030. The above-mentioned palindromic sequence is from the base number 3031 in the array table of the array number 1 to the base number 3213.
[0035] Moreover, the amino acid number -1 shows a signal sequence from the amino acid number -34 in the array table of the array number 2. The amino acid number 505 shows the amino acid sequence of PLD from the amino acid number 1 in the array table of the array number 2.
[0036] Setting to construction of a plasmid, genetic manipulation is Molecular cloning fundamentally. It carried out according to law. Moreover, a restriction enzyme is New England Biolabs. The DNA ligation kit ver2 (TAKARA SHUZO make) was used for ligation for the shrine thing.
[0037] The procedure of plasmid construction is shown in drawing 1 thru/or drawing 3 . first, the Actinomyces Stv.cinnamoneum origin (IFO12852) shown in drawing 1 pld gene promoterregion and a pld gene -- and -- It consists of a palindromic sequence of the pld gene downstream. a pld manifestation cassette -- PCR (Polymerase Chain (amplification) Reaction) It amplified by law. Generally, since GC content is high, an Actinomyces is promoterregion. It halved to the pld gene-palindromic sequence and PCR magnification was performed.
[0038] The primer was designed so that TTG (leucine) which is the initiation codon which is in a signal sequence part then might be set to ATG (methionine). As PCR magnification conditions, it is 30 cycle ****** about this magnification as 15 seconds, 30 seconds, and 7 minutes at 94 degrees C, 60 degrees C, and 68 degrees C in the temperature and time amount of thermal denaturation, annealing, and an expanding reaction, respectively.
[0039] The chromosome of Stv.cinnamoneum was used as a template. In order to use KOD-plus-DNA-Polymerase (Toyobo make) and to gather the magnification effectiveness of PCR magnification, GC-Melt (Clonetech shrine make) was added to magnification at the system of reaction so that it might become 10% of final concentration.

[0040] As a primer, for magnification of promoterregion 5 '-GGGTACCACGTCATGGCGGGTCTCTCTCGTCCG-3' and (S-PLD-38-Kpn I) 5'-TCTGCATGCTGCATCCTTAAACGAAGTAACGATTCCGCG-3' (AS-Pro-Sph I) it is used -- for magnification of a pld gene-palindromic sequence 5 '-ACAGCATGCTCCGCCACCGGCTCCGCCGTTTACACCGCT-3' and (S-Sig-Sph I) 5'-GGTAAGCTTGGTACCATTTCCTCGCTGGTCGGTTCGGGGCCAGCGCAT-3' (AS-Hin dIII and Kpn I-3213) were used.
[0041] The fragmentation of the promoterregion amplified by the PCR method is Escherichia coli after carrying out ligation to pUC18 which cut with the restriction enzyme Kpn I and the restriction enzyme Sph I, and was cut with the same enzyme. A transformation is carried out to Nova Blue and it is plasmid pUC18-promoter. It obtained.
[0042] On the other hand, the fragmentation of a pld gene-palindromic sequence is restriction enzymes Sph I and Hin dIII. It is Escherichia coli after carrying out ligation to pUC19 which cut to the site and was cut with the same enzyme. The transformation was carried out to Nova Blue and plasmid pUC19-pld was obtained.
[0043] Plasmid pUC18-promoter And the gene sequence of plasmid pUC19-pld was checked by the DNA sequencer (PE-biosystem gene analyzer 310).
[0044] Next, as shown in drawing 2 , they are a pUC19-pld plasmid to the restriction enzymes Sph I and Hin dIII. The fragmentation of a pld gene-palindromic sequence is started and, similarly they are restriction enzymes Sph I and Hin dIII. Cut pUC18-promoter It is Escherichia coli after carrying out ligation. A transformation is carried out to Nova Blue and it consists of a promoterregion-pld gene-palindromic sequence. Plasmid pUC18-promoter-pld which has a pld manifestation cassette It obtained.
[0045] On the other hand, as shown in drawing 3 , it is a shuttle vector pUC702. It prepared. pUC702 The vector pUC19 for Escherichia coli, and vector pIJ702 for Actinomyceses It is what was combined to Sac I and a Kpn I site, and prepared according to the approach of a publication to the following reports (report name: Molnar et al., J.Ferment.Bioeng., 72, and 368-372 (1991)).
[0046] Then, pUC18-promoter-pld From a plasmid The above-mentioned shuttle vector pUC702 which started the fragmentation containing a pld manifestation cassette with the restriction enzyme Kpn I, and was similarly cut with the restriction enzyme Kpn I It is Escherichia coli after carrying out ligation. A transformation is carried out to Nova Blue and it is plasmid pUC702-promoter-pld. It obtained. This plasmid pUC702-promoter-pld About whether the manifestation cassette was inserted in the right sense, it checked by the DNA sequencer.
[0047] (3) The Actinomyces (Stv.cinnamoneum) origin without promoterregion of the construction above of a pUC702-mature pld plasmid (IFO12852) Considering a pld gene expression vector as an example of a comparison, it is a shuttle vector pUC702. It used, and as shown in drawing 3 thru/or drawing 5 , it built.
[0048] First, the pld gene was made to amplify by two steps of PCR methods, as shown in drawing 4 . As conditions for First PCR, the temperature and time amount of thermal denaturation, annealing, and an expanding reaction are made into 45 seconds, 45 seconds, and 4 minutes at 98 degrees C, 52 degrees C, and 72 degrees C, respectively, and it is 25 cycle ****** about this magnification. The chromosome of Stv.cinnamoneum was used as a template. Using Pyrobest-DNA-polymerase (TAKARA SHUZO make), GC-Melt (Clonetech shrine make) was added to magnification at the system of reaction so that it might become 10% of final concentration.
[0049] As a primer for magnification, 5 '-CCCGGGAGCTGATAGCTTCTCCGCGTTGATCTTCC-3' (Genome-S) and 5'-CCATGATTACGAATTCCCGGGGATCTTGGT-3' (Genome2-AS) was used. The product after First PCR was used for the conditions of Second PCR as a template, and 5 '-TCGGAATTCGAGGTACCATGCTCCGCCACCGGCTCCGC-3' (Eco-Kpn-Sig-FW-PLD) and 5'-GCAGGTACCCCCCCTTGGCCGCGATTCCCG-3' (Kpn-Palind-RV-PLD) was used for them as a primer for magnification. The other magnification conditions are the same as that of First PCR.

[0050] Then, it is Escherichia coli after carrying out ligation to pUC19 which cut the amplified fragmentation (a signal sequence-mature pld gene-palindromic sequence is included) with the restriction enzyme Kpn I, and was cut with the same enzyme, as shown in drawing 5 after PCR. The transformation was carried out to Nova Blue and plasmid pUC19-pld was obtained. This plasmid The array of a pld gene was checked in the DNA sequencer.
[0051] pUC19-pld The plasmid was cut by Kpn I and the fragmentation (fragment DNA) containing a signal sequence-mature pld gene-palindromic sequence was collected. Shuttle vector pUC702 which was cut with the same enzyme and which is shown in drawing 3 It is Escherichia coli after carrying out ligation. By carrying out a transformation to Nova Blue, it is plasmid pUC702-pld. It obtained. This pUC702-pld Plasmid About whether the pld gene part was inserted in the right sense, it checked by the DNA sequencer.
[0052] (4) The transformation which the protoplast preparation Actinomyces protoplast of an Actinomyces prepares and mentions later was performed according to the publication of "GeneticManupulation of Streptomyces" mentioned above. as an Actinomyces -- a streptomyces Ribi dance (1326 shares of Streptomyces lividans), the following, and S.lividans -- outlining -- it was used. S. 5 mL which sterilized lividans Inoculation was carried out to the TSB culture medium, and seed culture was performed for two - three days at 30 degrees C.
[0053] To then, a Sakaguchi flask 150 mL The YEME culture medium and the above-mentioned stainless steel coil were put in, inoculation of the seed culture liquid 1mL was carried out, and main culture was performed at 30 degrees C for 36 hours to 40 hours. The above-mentioned YEME culture medium is Difco yeast extract 3 g/L, Difco bacto-peptone 5 g/L, Difco malt extract 3 g/L, glucose 10 g/L, and sucrose 340 g/L. MgCl2.6H2O(2.5M) 2 mL/L after containing and sterilizing with an autoclave Addition and glycine(20%) 25 mL/L It contains.
[0054] The culture medium which contains the cultivated fungus body after culture termination (S. before the coloring matter of lividans becomes red) was taken out, and 3000 rpm and centrifugal actuation for 10 minutes were performed to the above-mentioned culture medium. It is 50 mL about the fungus body which remained except for supernatant liquid from the culture medium by which centrifugal was carried out. The sucrose solution washed twice 10.3%.
[0055] To the fungus body after washing, it is 15 mL. The fungus body suspension which added the lysozyme (lysozyme) solution (2 mg/mL inLbuffer, filter sterilization) was prepared until it became, and the above-mentioned fungus body suspension was kept warm for 30 minutes at 30 degrees C. The above-mentioned fungus body suspension was stirred well [whenever precipitate arises].
[0056] Then, it is 15 mL to the above-mentioned fungus body suspension. It filtered by the cotton wool yarn which added Pbuffer, and stirred well, then sterilized the above-mentioned fungus body suspension. 3000 rpm and centrifugal actuation for 7 minutes were performed for the filtrate after filtration.
[0057] Pbuffer was added to the fungus body obtained by removing supernatant liquid from the filtrate by which centrifugal was carried out. In measurement by the haemacytometer, the amount which adds Pbuffer was adjusted so that it might be set to four to 5x109/mL. The fungus body protoplast suspension solution prepared such was poured distributively in the sterilization tube, and was saved at -70 degrees C.
[0058] L (Lysis) buffer sucrose(10.3%) 100 mL, TES buffer(5.73 %, pH7.2)10 mL, K2SO4 (2.5 %) 1 mL and trace element solution 0.2 mL, KH2PO4 (0.5%) 1 mL and MgCl2.6H2O(2.5M) 0.1 mL, It is a lysozyme (product made from NAKARAI) just before use to the solution which added distilled water to CaCl2 (2.5M) 1 mL, set to 1 L, and carried out autoclave sterilization 2 mg/mL It adds so that it may become.
[0059] P(protoplast) buffer sucrose 10.3 g, K2SO4 0.025 g, MgCl2.6H2O 0.202 g, trace element solution 0.2 mL It melts with distilled water and is 80 mL. As opposed to what carried out and carried out autoclave sterilization KH2PO4 (0.5%) 1 mL [finishing / autoclave sterilization / apart from this respectively], CaCl2.2H2O(3.68 %) 10 mL and TES buffer(product made from NAKARAI,

and TES what dissolved powder so that it might become 5.73%, and adjusted pH to 7.2)10 mL It adds, respectively.
[0060] trace element solution dissolves each in distilled water so that it may become ZnCl2 40 mg/L, FeCl3.6H2O 200 mg/L, CuCl2.2H2O 10 mg/L, MnCl2.4H2O 10 mg/L, Na2B4O7.10H2O 10 mg/L, and 6(NH4) Mo7O24.4H2O 10 mg/L.
[0061] (5) S.lividans which is the Actinomyces formed into the PUROTO pro strike as a transformation host of an Actinomyces as mentioned above was used. As mentioned above, the frozen object of the prepared suspension solution of the protoplast of S.lividans was thawed quickly, and 3000 rpm and centrifugal actuation for 7 minutes were performed at the room temperature. Then, plasmid solution given in (2) mentioned above 20 muL next, Tbuffer of 0.5 mL was added and pipetting was performed quickly.
[0062] Tbuffer was added, Pbuffer of 5 mL was added within 3 minutes, and centrifugal actuation (for 3000 rpm and 30 seconds) was performed lightly. Except for supernatant liquid, to the residue which precipitated, Pbuffer of 0.5 mL was added, and it applied to homogeneity mostly on the front face of every [0.1 mL] and each R2YE plate, and was kept warm at 30 degrees C.
[0063] every applied one day - two days after -- a R2YE plate -- receiving -- Tiostreptone (the Wako Pure Chem make, 50 mg/mL in DMSO as Stock solution) Final concentration It added so that it might become 500microg/mL. 0.7% soft agar (product made from NAKARAI) 3 mL every -- it applied to homogeneity mostly and, similarly was kept warm at 30 degrees C, respectively. This completed the transformation (recombination microorganism) of Actinomyces S.lividans which is a host.
[0064] This recombination microorganism is deposited as name:Streptomyceslividans/pUC702-PLD (IFO 16465) to Institute for Fermentation, Osaka (Osaka). Moreover, Actinomyces S.lividans (recombination microorganism) which operated to the above-mentioned (3) similarly, and carried out the transformation to it also about the plasmid solution of a publication was obtained.
[0065] T(Transformation) buffer 75 mL SEPEG1000 which dissolved 35.8 g PEG1000 in distilled water Solution, sucrose(10.3%) 25 mL, the above-mentioned trace element solution0.2 mL, and K2SO4 (2.5 %) 1 mL are mixed. What carried out autoclave sterilization is taken 9.3 mL, and CaCl2 (5M) 0.2 mL [finishing / autoclave sterilization / separately] and Tris-maleic acid buffer 0.5 mL are added. Above-mentioned Tris-maleic acid buffer Tris buffer of 1 M It is a malic acid (maleic acid) about pH of a solution. 8.0 It adjusts.
[0066] An R2YE plate is an R2YE culture medium. 100 mL It receives and is 2.2 g. Autoclave sterilization of Difco Bacto agar is added and carried out. KH2PO4 (0.5%) 1 mL [finishing / autoclave sterilization / separately before the agar solidifies], CaCl2.2H2O(5M) 0.4 mL, L-proline (20%) 1.5mL, and NaOH(1 N) 0.7 mL are added, and specified quantity impregnation is carried out and it considers as an R2YE plate at a petri dish [finishing / sterilization]. An R2YE culture medium is sucrose 103 g, K2SO4 0.25 g, MgCl2.6H2O 10.12 g, glucose 10 g, Difco casaminoacids 0.1 g, trace element solution 2 mL, Difco yeast extract 5 g, and TES buffer 5.73 g. It dissolves in distilled water 1 L, and prepares.
[0067] (6) Recombination S.lividans obtained by the approach to have carried out culture **** of the recombination S.lividans (recombination microorganism) was cultivated using the aeration stirring mold culture apparatus. First, the above-mentioned Tiostreptone (final concentration 5microg/mL) was added. TSB culture medium 5 mL Inoculation was carried out to the put-in test tube from the R2YE plate, and it cultivated during the 2nd - the 3rd at 30 degrees C. To furthermore, a Sakaguchi flask TSB culture medium 100 mL Tiostreptone (final concentration g/mL of 5micro) is added, and it is the seed culture liquid of the above-mentioned test tube. 1 mL Inoculation was carried out and it cultivated during the 2nd - the 3rd at 30 degrees C further.
[0068] As main culture, it is a TSB culture medium to an aeration stirring mold culture apparatus (the Able, Inc. make, jar fermenter of 2 L (BMJ-02PI)). 1 L and Tiostreptone (final concentration 5microg/mL) are added, and it is the seed culture liquid of a Sakaguchi flask. Inoculation of the 50

mL was carried out. Culture is the culture temperature of 28 degrees C, pH=7.0, DO(dissolved oxygen concentration) = 2 ppm, and quantity of airflow. It carried out by setting it as 2 vvm and controlling.

[0069] (7) If the choline generated when the activity measurement PC of PLD (it is also called phosphatidylcholine and lecithin (Lecithin)) is made to hydrolyze by PLD is made to react to choline oxidase, a peroxidase, and a phenol, red quinone coloring matter will be generated. The activity of Above PLD was measured by measuring this coloring matter with a spectrophotometer using choline esterase B-Test Wako (Wako Pure Chem make). Suppose that the definition of the enzyme activity of one unit (U) is separated in the choline of 1micro mol (mol) in 1 minute.

[0070] First, a reaction substrate mixed solution is prepared. It is the mixed presentation per tube in the above-mentioned reaction substrate mixed solution Tris-HCl (pH=7.4)20 of H2O 40microL and 200 mM muL and 10mM CaCl2 10microL and 1%Triton X-100 10 muL and Lecithin Emulsion 10microL of 50 mg/mL It carried out. Above Lecithin Emulsion Lecithin 500 mg obtained from the egg 1 mL It dissolves in diethylether and is with water. 10 mL is made to carry out ultrasonic suspension of what carried out the scalpel rise.

[0071] By the activity measurement of PLD, it is a PLD enzyme solution. 10 muL It receives and is an above-mentioned reaction substrate mixed solution. 90 muL It mixed, stirred well and was made to react for 10 minutes at 37 degrees C: EDTA of 0.5 mM was added after the reaction and the reaction was stopped. Next, substrate enzyme agent 500 (choline oxidase, a peroxidase, phenol) muL In addition, it is kept warm for 5 minutes at 37 degrees C, and is the reaction stop solution 500. muL In addition, the reaction was stopped.

[0072] Next, a calibration curve is created. They are water and the criteria liquid of choline esterase B-Test Wako, respectively 150 muL 0 muL and 140 muL 10microL and 130 muL 20microL and 120 muL 30microL It mixed and those absorbances (wavelength 505 nm) were measured with the spectrophotometer, respectively. The calibration curve was created noting that the value of each absorbance was equivalent to the PLD activity used as 0 U/L, 500 U/L, 1000 U/L, and 1500 U/L. The PLD activity in a sample was determined using this calibration curve.

[0073] (8) Electrophoresis method (SDS-PAGE)

In order to check the secretion manifestation of PLD, SDS-PAGE electrophoresis was performed using 12.5% of separation gel. It sets to electrophoresis and is a culture-medium sample. 20 muL It used. Dyeing of gel was performed using coloring matter (Coomassie Brilliant Blue). The above-mentioned electrophoresis was performed in reference (Garfin et al., Methods Enzymol.182, and 425-441 (1990)) based on the approach of a publication.

[0074] (9) The purification of PLD by which secretory production was carried out into the purification culture medium of PLD is pH6. The cation exchange resin (Macroprep high S (Bio-Rad make etc.)) which can be set performed. Elution was performed by the stepwise elution (0.5 M) or the linear concentration gradient method (0-0.5 M) of a sodium chloride. Moreover, the effectiveness that heparin affinity chromatography was also equivalent was shown.

[0075] Artificers are Stv.cinnamoneum (IFO 12852). Origin Cloning of a pld gene was performed and the efficient production process using various gene expression systems of PLD rearrange and according to a microorganism was found out.

[0076] First, in before, although this invention persons tried construction of the manifestation system using Escherichia coli and yeast (Pichia pastoris) as a manifestation system of PLD using a recombination vector, as for the case of Escherichia coli, in the case of 1/20 and yeast, only one fourth of manifestation active masses were able to check to Stv.cinnamoneum of a wild strain with the highest manifestation activity ability. However, also by each above ****, since the volume of PLD as protein itself carried out the extensive check, since it was checked that PLD of the Actinomyces origin forms the right spacial configuration of a natural mold, it was considered that the manifestation active mass became low by these manifestation systems.

[0077] Although Yamane and others was also developing the secretory production system in

Escherichia coli using PLD of the Actinomyces (Streptomyces antibioticus) origin, the manifestation active mass was about 3500 U/L extent at the maximum (Iwasaki et al., J.Ferment.Bioeng., 79, and 417-421 (1995)). Therefore, it was thought that development of the host-vector system of an Actinomyces was very important for development of the high secretory production system of Actinomyces PLD.

[0078] then, S.lividans whose this invention persons are kinds of an Actinomyces -- a host -- carrying out -- the Stv.cinnamoneum origin the manifestation system (shown in pUC702-pld, drawing 3 , or drawing 5) incorporating a pld gene -- further -- The manifestation system (shown in pUC702-promoter-pld, drawing 1 , or drawing 3) which incorporated promoterregion further was built to the pld gene. The transformation of the built class substitute plasmid was carried out to S.lividans which is an Actinomyces, respectively, and each S.lividans which carried out transformation was cultivated with the test tube.

[0079] Consequently, S.lividans/pUC702-pld In the manifestation system, although only the amount of manifestations equivalent to Stv.cinnamoneum of a wild strain was able to be checked, by the manifestation system (test tube culture 20000 U/L) of S.lividans/pUC702-promoter-pld, the remarkable big amount of manifestations (the amount of about 15 times) was able to be checked to Stv.cinnamoneum (test tube culture 1300 U/L) of a wild strain.

[0080] Therefore, the promotor concerning this invention is very powerful in the product from fungus body exogenous of protein, such as PLD for which it asks, and it was thought in the manifestation of various recombination protein including PLD that he was very effective.

[0081] Then, examination about optimization of the culture condition of this manifestation system S.lividans/pUC702-promoter-pld was performed using the aeration stirring mold cultivation tank. As conditions, to the above-mentioned TSB culture medium, it is making initiation addition glucose concentration into 0 g/l, 5 g/l, 15 g/l, and 30 g/l, and controlling it into the culture temperature of 28 degrees C, pH=7.0, and DO(dissolved oxygen concentration) = 2 ppm, respectively, and cultivated, respectively.

[0082] As shown in drawing 6 , when not adding a glucose, the activity of the secretion PLD in a culture medium is abbreviation. According to [although it was 20000 U/L as it is shown in drawing 7 thru/or drawing 9] the increment in the addition of a glucose, a PLD volume increases and is a glucose. 30 g/l By adding, it is abbreviation in a culture medium. It turns out that secretory production of the PLD activity of 30000 U/L can be carried out. This PLD activity is about 20 times the amount of manifestations of this compared with the PLD activity (the same TSB culture medium 1500 U/L) of Stv.cinnamoneum of a wild strain, and is about 15 times the amount of manifestations of this compared with the PLD activity (it optimizes, although it differs, and a culture medium is 2000 U/L by the case) of Stv.cinnamoneum of a wild strain.

[0083] However, it is initiation glucose concentration 30 g/l Even if it exceeded and having been enlarged, although cell mass increased, the volume of PLD did not increase. This was considered to be based on the effect of the catabolite repression resulting from existence of a superfluous glucose. The above-mentioned catabolite repression means that composition of many enzymes is checked with metabolite, such as catabolite repression, i.e., a glucose etc.

[0084] Furthermore, glucose concentration of initiation 15 g/l About the case, by electrophoresis (SDS-PAGE), the purity of PLD by which secretory production was carried out into the culture medium was checked, as shown in drawing 10 . In CBB coloring matter dyeing, contamination protein was hardly seen but PLD was able to check as a single band mostly.

[0085] It is abbreviation when the quantum of the PLD protein by which secretory production was carried out into this culture medium was carried out from the band of SDS-PAGE. It was 75 mg/L. It is abbreviation when the activity of PLD is calculated from this quantum value. It became 400 U/L. Before, this invention persons were the activity of the natural mold PLD which performed purification from Stv.cinnamoneum, and 468 U/L (Ogino et wl., J.Biochem., 125, and 263-269 (1999)). From this, PLD obtained in this invention had the value very near PLD of a natural mold.

[0086] Therefore, by the manufacture approach of PLD (protein) concerning this invention, PLD in a culture medium is high concentration, and it is a high grade, and moreover, since the activity of obtained PLD is also very close to PLD of a natural mold, it turns out that all of the trouble in various kinds of conventional manifestation systems including Escherichia coli were solvable.
[0087] Moreover, since the PLD purity by which the manufacture approach of PLD concerning this invention is secreted in a culture medium is high, after fungus body removal, with the ion exchange chromatography which used one step of ion exchange column, since separation recovery of PLD was possible, it could refine Above PLD very quickly and cheaply, and has mitigated the production cost of Above PLD.
[0088] as mentioned above, the PLD manifestation system of the Actinomyces (recombination microorganism) origin which included the promotor of the Actinomyces origin which this invention persons found out in the vector is very close to a natural mold -- high -- the fact that activity PLD can be mass-produced with a high grade and a high concentration gestalt in a culture medium (that is, outside of a fungus body) shows that it is a very effective PLD manifestation system.
[0089] Thus, there are few manifestation systems which can carry out secretory production of the recombination protein to a high grade into a culture medium conventionally, and the application to various kinds of useful protein production other than Above PLD is assumed. That is, the manifestation cassette (promotor-signal sequence-palindromic sequence) of the vector (plasmid) concerning this invention was assumed to be a very effective thing in the secretory production of various useful protein including the secretory production of the useful protein of the various Actinomyces origins, such as cholesterol oxidase.
[0090] Moreover, S.lividans/pUC702-promoter-pld which is the recombination microorganism of this invention Like Stv.cinnamoneum (Fukuda et al., Biotechnol.Lett., 18, 951-956 (1996)) Since efficient immobilization to a microorganism maintenance particle (BSPs) can be performed, A mass production of useful protein including PLD using an immobilized cell is also possible, the productivity of useful protein can be raised by leaps and bounds, and cheap-ization of the above-mentioned useful protein can be attained.

[Translation done.]

* NOTICES *

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is the explanatory view of DNA including the promoter of this invention for PLD production.
[Drawing 2] It is the explanatory view of a vector including the above-mentioned promoter.
[Drawing 3] It is the explanatory view of other precursors for creating the above-mentioned vector.
[Drawing 4] It is the explanatory view of DNA for PLD production which excluded the above-mentioned promotor for a comparison.
[Drawing 5] It is the explanatory view of the vector containing DNA for the above-mentioned PLD production.
[Drawing 6] It is the glucose additive-free graph incorporating the vector shown in drawing 2 by the recombination microorganism of this invention which shows the productivity of PLD.
[Drawing 7] Glucose concentration incorporating the vector shown in drawing 2 by the recombination microorganism of this invention It is the graph in 5 g/L which shows the productivity of PLD.
[Drawing 8] Glucose concentration incorporating the vector shown in drawing 2 by the recombination microorganism of this invention 15 g/L It is the graph which can be set and which shows the productivity of PLD.
[Drawing 9] Glucose concentration incorporating the vector shown in drawing 2 by the recombination microorganism of this invention 30 g/L It is the graph which can be set and which shows the productivity of PLD.
[Drawing 10] Glucose concentration incorporating the vector shown in drawing 2 by the recombination microorganism of this invention 15 g/L In the culture which can be set, it is the drawing substitution photograph of SDS-PAGE in which PLD in the culture medium in a stationary phase is shown.

[Translation done.]

(19)日本国特許庁（ＪＰ）　(12)**公開特許公報**（A）　(11)特許出願公開番号
特開2002－51780
(P2002－51780A)
(43)公開日　平成14年２月19日(2002.2.19)

(51)Int.Cl.⁷	識別記号	ＦＩ	ﾃｰﾏｺｰﾄﾞ(参考)
Ｃ１２Ｎ　15/09	ＺＮＡ	Ｃ１２Ｎ　1/21	４Ｂ０２４
1/21		Ｃ１２Ｐ　21/02	Ｃ　４Ｂ０６４
Ｃ１２Ｐ　21/02		Ｃ１２Ｒ　1:625)	４Ｂ０６５
// (Ｃ１２Ｎ　15/09	ＺＮＡ	(Ｃ１２Ｎ　1/21	
Ｃ１２Ｒ　1:625)		Ｃ１２Ｒ　1:465)	

審査請求　未請求　請求項の数13　ＯＬ　（全 17 頁）　最終頁に続く

(21)出願番号　特願2000－239132(P2000－239132)

(22)出願日　平成12年８月７日(2000.8.7)

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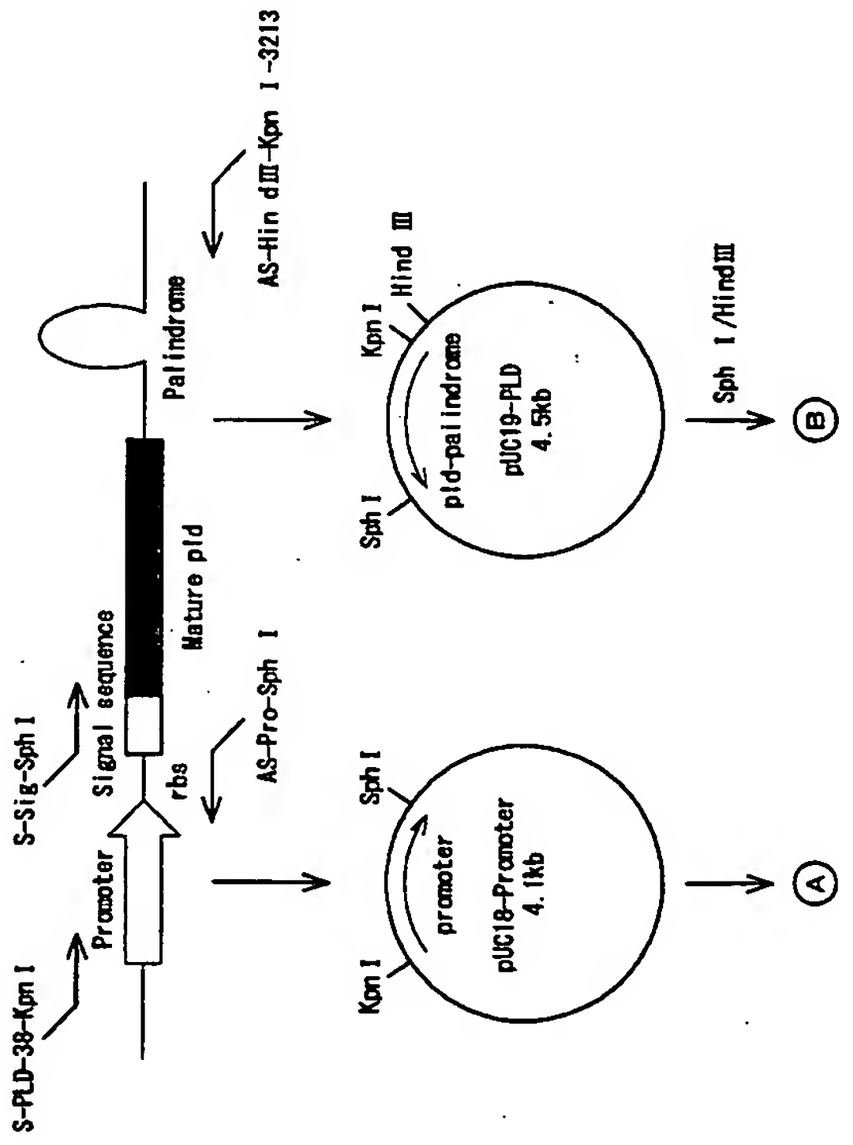
(54)【発明の名称】　プロモーター、それを有するベクター、および組換え微生物、並びにタンパク質の製造方法

(57)【要約】
【課題】　ホスフォリパーゼＤなどのタンパク質を効率良く生産できる、プロモーター、それを用いたベクター、および組換え微生物、並びにタンパク質の製造方法を提供する。
【解決手段】　ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来の塩基配列であり、タンパク質生産のためのタンパク質用塩基配列の上流側に結合されて、上記タンパク質生産能の向上活性を有しているプロモーター、それを用いたベクター、および組換え微生物、並びにタンパク質の製造方法。



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【特許請求の範囲】
【請求項１】ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来の塩基配列であり、タンパク質生産のためのタンパク質用塩基配列の上流側に結合されて、上記タンパク質生産能の向上活性を有していることを特徴とするプロモーター。
【請求項２】配列番号１の配列表における、塩基番号３８から塩基番号１４０７までの塩基配列を有する、または、上記塩基配列において１もしくは数個の塩基配列が欠失、置換もしくは付加された塩基配列からなり、タンパク質生産のためのタンパク質用塩基配列の上流側に結合されて、上記タンパク質生産能の向上活性を有していることを特徴とするプロモーター。
【請求項３】請求項１または２記載のプロモーターと、上記プロモーターの下流側にタンパク質生産のためのタンパク質用塩基配列とを有していることを特徴とするベクター。
【請求項４】タンパク質用塩基配列は、ホスフォリパーゼＤの生成用であることを特徴とする請求項３記載のベクター。
【請求項５】タンパク質用塩基配列は、ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来のものであることを特徴とする請求項４記載のベクター。
【請求項６】タンパク質用塩基配列は、配列番号１の配列表における、塩基番号１５１２から塩基番号３０３０までの塩基配列、または上記塩基配列において１もしくは数個の塩基配列が欠失、置換もしくは付加された、タンパク質生産能を備えた塩基配列であることを特徴とする請求項５記載のベクター。
【請求項７】タンパク質用塩基配列の下流側に、タンパク質の翻訳を停止するためのパリンドローム配列を有していることを特徴とする請求項３ないし６の何れかに記載のベクター。
【請求項８】パリンドローム配列は、配列番号１の配列表における、塩基番号３０３１から塩基番号３２１３までの塩基配列、または上記塩基配列において１もしくは数個の塩基配列が欠失、置換もしくは付加された、タンパク質の翻訳を停止する機能を備えた塩基配列であることを特徴とする請求項７記載のベクター。
【請求項９】請求項３ないし８の何れかのベクターが宿主内に組み込まれていることを特徴とする組換え微生物。
【請求項１０】宿主は、放線菌であることを特徴とする請求項９記載の組換え微生物。
【請求項１１】宿主は、ストレプトマイセス・リヴィダンスであることを特徴とする請求項１０記載の組換え微生物。
【請求項１２】請求項９ないし１１の何れかに記載の組換え微生物を培養液中にて培養して、タンパク質用塩基配列に基づくタンパク質を生産することを特徴とするタンパク質の製造方法。

2

【請求項１３】培養液に、グルコースを添加することを特徴とする請求項１２記載のタンパク質の製造方法。
【発明の詳細な説明】
【０００１】
【発明の属する技術分野】本発明は、ホスフォリパーゼＤ（phospholipase D 、以下、ＰＬＤという）などの酵素のタンパク質を効率良く生産できる、プロモーター、それを用いたベクター、および上記ベクターを組み込んだ組換え微生物、並びに上記組換え微生物によるタンパク質の製造方法に関するものである。
【０００２】
【従来の技術】ＰＬＤは、高いエステル交換反応活性を有するため、有用な各種のリン脂質合成反応への応用が可能なものであり、その応用範囲を広げるために、ＰＬＤの工業化つまり大量生産による低廉化が期待されている。
【０００３】このようなＰＬＤの有用性から、ＰＬＤを大量分泌生産する微生物のスクリーニングが広範に行われてきた。そのスクリーニングの結果、現時点では、放線菌群のみが大量のＰＬＤを分泌生産することが知られている。
【０００４】それら放線菌群の中でも、ストレプトバーティシリウム・シンナモニウム(ＩＦＯ12852) (Streptoverticillium cinnamoneum 、以下、Stv. cinnamoneum （ＩＦＯ12852)と略記する）は、現時点において、分泌ＰＬＤ活性の最も高い菌株であることが判っている(Nakajima et al., Biotechnol. Bioeng., Vol.44, 1193-1198 (1994))。
【０００５】上記のＩＦＯ番号は、微生物の国内寄託機関である、財団法人醗酵研究所（所在、大阪市）にて入手できる微生物のカタログ番号である。なお、ストレプトバーティシリウムについては、分類上、ストレプトマイセス（Streptomyces）に分類されることがある。
【０００６】また、従来、野性株のStv. cinnamoneumについて、肉エキスをベースとする培地を用い、培地組成や培養条件を種々変更して、ＰＬＤの発現活性量を最大化することが試みられたが、その発現活性は最大でも2000単位／リットル（以下、U/L と略記する）程度であった。本明細書では、単位中のL は、特に断らないかぎり、1000 cm^3を示すものとする。
【０００７】また、得られたＰＬＤの精製が容易な、より夾雑タンパク質の少ない TSB培地を用いた場合では、野性株のStv. cinnamoneumにおける、ＰＬＤの発現活性量はさらに低く、1500 U/L程度にとどまっていた。
【０００８】
【発明が解決しようとする課題】ところが、上記従来では、培地中に分泌されるＰＬＤの含量割合が、まだ低いことから、得られたＰＬＤは、培地からＰＬＤを精製するのに手間取り、ＰＬＤの生産コストが高くなることに

よって高価なものとなり、ＰＬＤの適用可能範囲が制限されるという問題を生じている。

【０００９】

【課題を解決するための手段】本発明のプロモーター(promoter)は、以上の課題を解決するために、ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来の塩基配列であり、ＰＬＤ等のタンパク質生産のためのタンパク質用塩基配列(mat peptide) の上流側に結合されて、上記タンパク質生産能の向上活性を有していることを特徴としている。

【００１０】上記プロモーターとしては、配列番号１の配列表における、塩基番号３８から塩基番号１４０７までの塩基配列を有する、または、上記塩基配列において１もしくは数個の塩基配列が欠失、置換もしくは付加された塩基配列からなり、タンパク質生産のためのタンパク質用塩基配列の上流側に結合されて、上記タンパク質生産能の向上活性を有しているものであってもよい。

【００１１】上記の構成によれば、ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来の上記塩基配列を有することにより、ＰＬＤなどのタンパク質の生産能を向上させることができる。

【００１２】本発明のベクターは、以上の課題を解決するために、上記プロモーターと、上記プロモーターの下流側にタンパク質生産のためのタンパク質用塩基配列とを有していることを特徴としている。

【００１３】上記ベクターでは、タンパク質用塩基配列は、ＰＬＤの生産用であってもよく、また、ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来のものであってもよい。

【００１４】さらに、上記タンパク質用塩基配列は、配列番号１の配列表における、塩基番号１５１２から塩基番号３０３０までの塩基配列、または上記塩基配列において１もしくは数個の塩基配列が欠失、置換もしくは付加された、タンパク質生産能を備えた塩基配列であってもよい。

【００１５】上記の構成によれば、上記プロモーターを有することにより、例えば、ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来の、ホスフォリパーゼＤの生成用等のタンパク質用塩基配列によるタンパク質生産能を向上させることが可能となる。

【００１６】上記ベクターにおいては、タンパク質用塩基配列の下流側に、タンパク質の翻訳を停止するためのパリンドローム配列(terminator)を有していることが好ましい。上記構成によれば、ＰＬＤなどのタンパク質の生産能を向上させることができる。

【００１７】本発明の組換え微生物は、前記の課題を解決するために、上記の何れかのベクターが宿主内に組み込まれていることを特徴としている。上記組換え微生物では、宿主は、放線菌であってもよく、また、ストレプトマイセス・リヴィダンスであってもよい。

【００１８】上記組換え微生物では、ベクターに組み込まれたタンパク質用塩基配列に沿ったタンパク質の生産能が、組み込まれたプロモーターによって、向上しており、上記タンパク質を効率よく、かつ、培地中にて高純度にて生産できる。

【００１９】本発明のタンパク質の製造方法は、前記の課題を解決するために、上記の何れかに記載の組換え微生物を培養液中にて培養して、タンパク質用塩基配列に基づくタンパク質を生産することを特徴としている。上記製造方法では、培養液に、グルコースを添加することが好ましい。

【００２０】上記方法によれば、ベクター中に、上記プロモーターが組み込まれていることによって、ＰＬＤなどのタンパク質を効率よく、培地中に高純度にて分泌して生産することができる。

【００２１】

【発明の実施の形態】本発明の実施の形態について図１ないし図１０に基づいて説明すれば、以下の通りである。

【００２２】（１）放線菌からＤＮＡ（ゲノム）の抽出放線菌からのＤＮＡ（ＰＬＤ生成用）抽出は、放線菌に関する遺伝子操作技術を扱った、「Genetic Manupulation of Streptomyces」(Hopwood,D.A., Bibb,M.J., Chater,K.F., Kieser,T., Bruton,C.J., Kieser,H.M., Lydiate,D.J., Smith,C.P., Ward,J.M., and Schremph,H. (1985) "Genetic Manupulation of Streptomyces: a Laboratory Manual", the John Innes Foundation, Norwich)に記載の方法に従って行った。

【００２３】放線菌（Stv. cinnamoneum（ＩＦＯ12852)）を、滅菌した 100 mL のトリプティクソイブロス培地（Tryptic Soy Broth 、以下、 TSB培地という）にて振とう培養を、温度30℃にて48時間行った。この振とう培養の際、培地中に、直径が２－３cm程の大きさのステンレスコイルを加えた。これにより、振とう培養中における、フロック状菌体の形成を抑制した。

【００２４】上記TSB 培地としては、pancreatic digest of casein 17.0 g/L, papaic digest of soybean mea l 3.0 g/L, dextrose 2.5 g/L, sodium chloride 5.0 g/L,dipotassium phosphate 2.5 g/L を含むDifco 社製のものを用いた。本明細書において、滅菌とは、飽和水蒸気下、121 ℃で20分間曝すことにより、培地等の溶液中に含まれる微生物を死滅させることである。

【００２５】培養終了後、得られた菌体を集菌し、ＴＥbuffer（10mM Tris-HCl (pH 8.0)、1 mM EDTA をオートクレーブにて滅菌したもの）にて洗浄を３回行った。洗浄後、5 mLのＴＥbufferに菌体を懸濁し、溶菌を促進させるために、リゾチーム、アクロモペプチダーゼを、それぞれ、2 mg/mL 、4 mg/mL となるように加え、30℃にて液体の粘性が高くなるまで保温した（約30分間）。

【００２６】その後、溶菌溶液に対し、プロナーゼ、 Ｅ

DTA 、SDS をさらに加え、37℃にて２時間保温した。保温終了後、溶菌溶液から、フェノール・クロロホルム抽出法、ポリエチレングリセロール沈殿法にて、用いた放線菌の染色体ＤＮＡをガラス棒にて取り出した。最後に、取り出した染色体ＤＮＡにＲNase 処理を施し、染色体ＤＮＡ（ゲノム）の抽出操作を終了した。本明細書では、ＰＬＤの生成に関する遺伝子については、 pld遺伝子という。

【００２７】次に、 pld遺伝子プロモーター領域およびバリンドローム領域のＤＮＡ配列の決定について説明する。

【００２８】上述の取り出した染色体ＤＮＡ（ゲノム）を制限酵素Ｓau 3A1 で切断した後、断片化させた断片ＤＮＡをそれぞれクローニングベクターｐＵＣ8 にライゲーションし、大腸菌Nova Blue (Novagen社製）に形質転換することにより、ゲノムライブラリーを構築した。

【００２９】ＰＬＤのアミノ酸配列解析の結果から、アミノ酸末端側の約25アミノ酸配列が確認されていたため、そのアミノ酸配列に相当するＤＮＡプローブを作成し、コロニーハイブリダイゼーションにより、 pld遺伝子の同定を行った。その結果、pld遺伝子を部分的に含む断片ＤＮＡを２種類取得した。

【００３０】一つは、ＰＬＤアミノ酸末端側の塩基配列と、その上流約１５００ベース（塩基）を含む断片ＤＮＡであり、他方は、ＰＬＤカルボキシル基末端側の塩基配列とその下流約１０００ベース（塩基）を含む断片ＤＮＡであった。それぞれの断片ＤＮＡの塩基配列をサンガー法により解析し、重複する塩基配列を元に、 pld遺伝子の地図（塩基配列）を構築した。

【００３１】解析した全塩基配列より、配列表に示すように、 pld遺伝子の下流側には、タンパク質翻訳を物理的に終了させる機能を有するパリンドローム配列が存在することが確認された。

【００３２】また、後述するように、上記 pld遺伝子を組み込んだ放線菌におけるＰＬＤの高い分泌発現活性量から、 pld遺伝子の上流側においては、強力な発現プロモーター配列が存在すると想定されたため、解析の終了した上流側約１０００ベース（塩基）の塩基配列の領域をプロモーター領域と設定した。上記プロモーター領域は、配列番号１の配列表における、塩基番号３８から塩基番号１４０７までである。

【００３３】（２）ｐＵＣ702-promoter－pld プラスミドの構築

pld発現カセットを有する放線菌用の pld分泌発現ベクター（ｐＵＣ702-promoter-pld）の調製を以下の手順にて行った〔図１ないし図３、並びに配列表を参照〕。 pld発現カセットとは、 pld遺伝子の上流側のプロモーター領域（プロモーターおよびリボソーム結合サイト）、 pld遺伝子（シグナル配列(sig peptide) とmature pld遺伝子(mat peptide) からなる）、および pld遺伝子の

下流側のパリンドローム配列とを有するものである。

【００３４】上記リボソーム結合サイトは、配列番号１の配列表における、塩基番号１３９６から塩基番号１４０３までである。上記シグナル配列は、配列番号１の配列表における、塩基番号１４０８から塩基番号１５０９までである。上記mature pld遺伝子は、シグナル配列を含まない分泌後のＰＬＤ配列に対応する部分であり、配列番号１の配列表における、塩基番号１５１０から塩基番号３０３０までである。上記パリンドローム配列は、配列番号１の配列表における、塩基番号３０３１から塩基番号３２１３までである。

【００３５】また、配列番号２の配列表における、アミノ酸番号－３４からアミノ酸番号－１は、シグナル配列を示す。配列番号２の配列表における、アミノ酸番号１からアミノ酸番号５０５は、ＰＬＤのアミノ酸配列を示す。

【００３６】プラスミドの構築において、遺伝子操作は、基本的にMolecular cloning 法に従って行った。また、制限酵素は、New England Biolabs 社製のものを、ライゲーションには、ＤＮＡライゲーションキットver2（宝酒造製）を用いた。

【００３７】図１ないし図３には、プラスミド構築の手順を示す。まず、図１に示す、放線菌Stv. cinnamoneum（ＩＦＯ12852)由来の pld遺伝子プロモーター領域、 pld遺伝子、および pld遺伝子下流側のパリンドローム配列からなる pld発現カセットをＰＣＲ(Polymerase Chain (amplification) Reaction) 法により増幅した。一般に、放線菌はＧＣ含量が高いために、プロモーター領域と pld遺伝子ーパリンドローム配列とに二分割して、ＰＣＲ増幅を行った。

【００３８】その時に、シグナル配列部分にある開始コドンであるTTG(ロイシン）を、ATG(メチオニン）になるようにプライマーを設計した。ＰＣＲ増幅条件として、熱変性、アニーリング、伸長反応の温度および時間をそれぞれ、例えば94℃、60℃、68℃で15秒、30秒、７分として、この増幅を30サイクル行った。

【００３９】テンプレートとしては、Stv. cinnamoneumの染色体を用いた。増幅には、ＫＯＤ-plus-ＤＮＡ-Polymerase （東洋紡製）を使用し、ＰＣＲ増幅の増幅効率を上げるために、反応系には終濃度10％となるように、ＧＣ-Melt （Clonetech 社製）を加えた。

【００４０】プライマーとしては、プロモーター領域の増幅には、5'-GGGTACCACGTCATGGCGGGTCTCTCTCGTCCG-3'（Ｓ－ＰＬＤ-38-Kpn I）および5'-TCTGCATGCTGCATCCTTAAACGAAGTAACGATTCCGCG-3' （ＡＳ－Pro－Sph I）を使用し、 pld遺伝子ーパリンドローム配列の増幅には、5'-ACAGCATGCTCCGCCACCGGCTCCGCCGTTTACACCGCT-3' （Ｓ－Ｓig－Ｓph I）および5'-GGTAAGCTTGGTACCATTTCCTCGCTGGTCGGTTCGGGGCCAGCGCAT-3'（ＡＳ－Hin dIII ・Kpn I-3213 ）を使用した。

【００４１】ＰＣＲ法で増幅したプロモーター領域のフラグメントは、制限酵素Ｋpn Iおよび制限酵素Ｓph Iで切断し、同一酵素で切断したｐＵＣ18とライゲーションした後に、大腸菌 Nova Blueに形質転換し、プラスミドｐＵＣ18-promoter を得た。

【００４２】一方、 pld遺伝子ーパリンドローム配列のフラグメントは、制限酵素Ｓph Iおよび、Ｈin dIII サイトで切断し、同一酵素で切断したｐＵＣ19とライゲーションした後に、大腸菌 Nova Blueに形質転換し、プラスミドｐＵＣ19-pldを得た。

【００４３】プラスミドｐＵＣ18-promoter およびプラスミドｐＵＣ19-pldの遺伝子配列は、ＤＮＡシーケンサー（ＰＥ-biosystem gene analyzer 310）により確認した。

【００４４】次に、図２に示すように、ｐＵＣ19-pldプラスミドから制限酵素Ｓph IおよびＨin dIII により、pld遺伝子ーパリンドローム配列のフラグメントを切り出し、同じく制限酵素Ｓph IおよびＨin dIII で切断したｐＵＣ18-promoter とライゲーションした後に、大腸菌 Nova Blueに対し形質転換し、プロモーター領域-pld遺伝子ーパリンドローム配列からなる pld発現カセットを有するプラスミドｐＵＣ18-promoter-pld を得た。

【００４５】一方、図３に示すように、シャトルベクターｐＵＣ702 を調製した。ｐＵＣ702 は、大腸菌用ベクターｐＵＣ19、および放線菌用ベクターｐＩＪ702 をＳac IおよびＫpn Iサイトで結合したもので、以下の報文に記載の方法に従って調製した（報文名：Molnar et al., J. Ferment. Bioeng., 72, 368-372 (1991)）。

【００４６】続いて、ｐＵＣ18-promoter-pld プラスミドから pld発現カセットを含むフラグメントを制限酵素Ｋpn Iにより切り出し、同じく制限酵素Ｋpn Iにより切断した上記シャトルベクターｐＵＣ702 とライゲーションした後に、大腸菌 Nova Blueに形質転換し、プラスミドｐＵＣ702-promoterーpld を得た。このプラスミドｐＵＣ702-promoterーpld の発現カセットが正しい向きに挿入されたか否かについては、ＤＮＡシーケンサーにより確認した。

【００４７】（３）ｐＵＣ702-mature pldプラスミドの構築

上記のプロモーター領域を持たない放線菌（Stv. cinnamoneum（ＩＦＯ12852)）由来の pld遺伝子発現ベクターを、比較例として、シャトルベクターｐＵＣ702 を用い、図３ないし図５に示すように構築した。

【００４８】まず、図４に示すように、二段階のＰＣＲ法により、 pld遺伝子を増幅させた。First ＰＣＲの条件として、熱変性、アニーリング、伸長反応の温度および時間を、それぞれ、例えば98℃、52℃、72℃で、45秒、45秒、４分とし、この増幅を25サイクル行った。テンプレートとしては、Stv. cinnamoneumの染色体を使用した。増幅には、Pyrobestー ＤＮＡ-polymerase(宝酒造

製）を用い、終濃度10%となるように、ＧＣ-Melt(Clonetech 社製）を反応系に加えた。

【００４９】増幅用のプライマーとしては、5'-CCCGGGAGCTGATAGCTTCTCCGCGTTGATCTTCC-3'（Genome-S）および5'-CCATGATTACGAATTCCCGGGGATCTTGGT-3'（Genome2-AS）を使用した。 Second ＰＣＲの条件は、テンプレートとしてFirst ＰＣＲ後の生成物を使用し、増幅用のプライマーとして、5'-TCGGAATTCGAGGTACCATGCTCCGCCACCGGCTCCGC-3'（Ｅcoー Kpnー Ｓ igー ＦＷー ＰＬＤ）および5'-GCAGGTACCCCCCCTTGGCCGCGATTCCCG-3'（Kpn-Palind-ＲＶー ＰＬＤ）を使用した。それ以外の増幅条件はFirst ＰＣＲと同様である。

【００５０】続いて、ＰＣＲ後、図５に示すように、増幅されたフラグメント（シグナル配列ーmature pld遺伝子ーパリンドローム配列を含む）を制限酵素Ｋpn Iで切断し、同一酵素で切断したｐＵＣ19とライゲーションした後に、大腸菌 Nova Blueに形質転換し、プラスミドｐＵＣ19-pldを得た。このプラスミドの pld遺伝子の配列をＤＮＡシーケンサーにて確認した。

【００５１】ｐＵＣ19ーpld プラスミドをＫpn Iで切断して、シグナル配列ーmature pld遺伝子ーパリンドローム配列を含むフラグメント（断片ＤＮＡ）を回収した。同一酵素で切断した、図３に示すシャトルベクターｐＵＣ702 とライゲーションした後に、大腸菌 Nova Blueに形質転換することで、プラスミドｐＵＣ702-pld を得た。このｐＵＣ702-pld プラスミドの pld遺伝子部分が正しい向きに挿入されたか否かについては、ＤＮＡシーケンサーにより確認した。

【００５２】（４）放線菌のプロトプラスト調製

放線菌プロトプラストの調製および後述する形質転換は、前述した「GeneticManupulation of Streptomyces」の記載に従って行った。放線菌としては、ストレプトマイセス・リヴィダンス（Streptomyces lividans 1326株）、以下、S.lividansと略記する）を使用した。S.lividansを滅菌した5 mLの TSB培地に植菌し、30℃にて２～３日間種培養を行った。

【００５３】続いて、坂口フラスコに 150 mL のYEME培地と、前述のステンレスコイルとを入れ、種培養液１mLを植菌し、36時間～40時間、30℃にて本培養を行った。上記YEME培地は、Difco yeast extract 3 g/L 、Difco bacto-peptone 5 g/L 、Difco malt extract 3 g/L、glucose 10 g/L、sucrose 340 g/L を含み、オートクレーブにて滅菌した後、 $MgCl_2 \cdot 6H_2O$(2.5M) 2 mL/L 添加、glycine(20%) 25 mL/L を含むものである。

【００５４】培養終了後（S.lividansの色素が赤くなる前）、培養された菌体を含む培地を取り出し、上記培地に対し3000 rpm、10分間の遠心操作を行った。遠心された培地から上清を除き、残った菌体を50 mL の10.3% sucrose溶液で２回洗浄した。

【００５５】洗浄後の菌体に、15 mL になるまでリゾチ

ーム(lysozyme)溶液（ 2 mg/mL in L buffer、フィルター滅菌）を加えた菌体懸濁液を調製し、上記菌体懸濁液を30℃にて30分間保温した。上記菌体懸濁液は、沈殿が生じる度によく撹拌した。

【００５６】その後、上記菌体懸濁液に対し、15 mL のP bufferを加え、よく撹拌し、続いて、上記菌体懸濁液を滅菌したコットンウールによりろ過した。ろ過後のろ液を、3000 rpm、７分間の遠心操作を行った。

【００５７】遠心されたろ液から上清を除くことにより得られた菌体に対しP bufferを加えた。P bufferを加える量は、血球計による測定において、４～５×１０^9/mLとなるよう調節した。そのように調製された菌体プロトプラスト懸濁溶液を、滅菌チューブに分注し、－70℃で保存した。

【００５８】L (Lysis) bufferは、sucrose(10.3%) 100 mL、TES buffer(5.73 %, pH7.2)10 mL、K_2SO_4 (2.5 %) 1 mL、trace element solution 0.2 mL 、KH_2PO_4 (0.5%) 1 mL、 $MgCl_2 \cdot 6H_2O$(2.5M) 0.1 mL 、$CaCl_2$ (2.5M) 1 mLに蒸留水を加えて１ Lとしオートクレーブ滅菌した溶液に対し、使用直前にリゾチーム（ナカライ製）を、2 mg/mL となるように加えたものである。

【００５９】P (protoplast)bufferは、sucrose 10.3 g、K_2SO_4 0.025 g 、 $MgCl_2 \cdot 6H_2O$ 0.202 g、trace element solution 0.2 mL を蒸留水で溶かし80 mL としてオートクレーブ滅菌したものに対し、これとは別に、それぞれオートクレーブ滅菌済の、KH_2PO_4 (0.5%) 1 mL、 $CaCl_2 \cdot 2H_2O$(3.68 %) 10 mL 、TES buffer(ナカライ製、TES 粉末を5.73%となるように溶解し、pHを7.2 に調整したもの) 10 mL をそれぞれ添加したものである。

【００６０】trace element solutionは、$ZnCl_2$ 40 mg/L 、 $FeCl_3 \cdot 6H_2O$ 200 mg/L 、$CuCl_2 \cdot 2H_2O$ 10 mg/L、$MnCl_2 \cdot 4H_2O$ 10 mg/L、 $Na_2B_4O_7 \cdot 10H_2O$ 10 mg/L 、$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 10 mg/Lとなるように、それぞれを蒸留水に溶解したものである。

【００６１】（５）放線菌の形質転換

宿主として、上述したようにプロトプロスト化した放線菌であるS.lividansを用いた。上述したように調製したS.lividansのプロトプラストの懸濁溶液の冷凍物を素早く解凍し、室温で3000 rpm、７分間の遠心操作を行った。続いて、前述した（２）に記載のプラスミド溶液を 20 μL 加え、次に、0.5 mLのT bufferを加えて素早くピペッティングを行った。

【００６２】T bufferを加えて３分以内に、5 mLのP bufferを加え、軽く遠心操作(3000 rpm、30秒間) を行った。上清を除き、沈殿した残渣に対し、0.5 mLのP bufferを加え、0.1 mLずつ、各R2YEプレートの表面にほぼ均一に塗布して30℃にて保温した。

【００６３】１日～２日後、塗布された各R2YEプレートに対し、Tiostreptone(和光純薬製、Stock solutionとして50 mg/mL in ＤＭＳＯ) を終濃度 500μg/mLとなるように加えた 0.7% soft agar（ナカライ製）を 3 mLずつ、それぞれ、ほぼ均一に塗布して、同じく30℃にて保温した。これにより、宿主である放線菌S.lividansの形質転換（組換え微生物）を完了した。

【００６４】この組換え微生物は、（財）発酵研究所（大阪）に対し、名称:Streptomyces lividans /pUC702-PLD（ＩＦＯ 16465）として寄託されているものです。また、前述の（３）に記載のプラスミド溶液についても同様に操作して形質転換した放線菌S.lividans（組換え微生物）を得た。

【００６５】T (Transformation)bufferは、75 mL の蒸留水に、35.8 g PEG1000を溶解したＳＥPEG1000 溶液と、sucrose(10.3%) 25 mL 、前述のtrace element solution0.2 mL 、および、K_2SO_4 (2.5 %) 1 mLとを混合して、オートクレーブ滅菌したものを、9.3 mLとり、別々にオートクレーブ滅菌済の、$CaCl_2$ (5M) 0.2 mL、Tris-maleic acid buffer 0.5 mLを加えたものである。上記のTris-maleic acid buffer は、 1 MのTris buffer 溶液のpHをリンゴ酸(maleic acid) にて8.0 に調整したものである。

【００６６】R2YEプレートは、R2YE培地 100 mL に対して、2.2 g のDifco Bacto agarを加えオートクレーブ滅菌し、そのagarが固化する前に、別々にオートクレーブ滅菌済の、KH_2PO_4 (0.5%) 1 mL、 $CaCl_2 \cdot 2H_2O$(5M) 0.4 mL 、L-proline(20%) 1.5mL 、NaOH(1 N) 0.7 mLを加え、滅菌済のシャーレに所定量注入して、R2YEプレートとしたものである。R2YE培地は、sucrose 103 g 、K_2SO_4 0.25 g、 $MgCl_2 \cdot 6H_2O$ 10.12 g、glucose 10 g、Difco casaminoacids 0.1 g 、trace element solution 2 mL 、Difco yeast extract 5 g 、および、TES buffer 5.73 g を蒸留水1 Lに溶解して調製したものである。

【００６７】（６）組換えS.lividans（組換え微生物）の培養

上述した方法により得られた組換えS.lividansを、通気撹拌型培養装置を用いて培養を行った。まず、前述のTiostreptone（終濃度 5μg/mL）を加えた TSB培地 5 mLを入れた試験管に、R2YEプレートから植菌し、30℃で培養を２日～３日間行った。さらに、坂口フラスコに TSB培地 100 mL と、Tiostreptone（終濃度5μg/mL）を加え、上記試験管の種培養液 1 mL を植菌し、さらに、30℃で培養を２日～３日間行った。

【００６８】本培養として、通気撹拌型培養装置（エイブル社製、２Ｌ のジャーファーメンター(BMJ-02PI)）に、 TSB培地 1 LとTiostreptone（終濃度 5μg/mL）とを加え、坂口フラスコの種培養液 50 mLを植菌した。培養は、培養温度28℃、pH=7.0、DO（溶存酸素濃度）＝ 2 ppm、通気量 2 vvmに設定して制御することで行った。

【００６９】（７）ＰＬＤの活性測定

ＰＣ（ホスファチジルコリン、レシチン(Lecithin)ともいう）をＰＬＤによって加水分解させたときに生成する

コリンを、コリンオキシダーゼ、ペルオキシダーゼとフェノールに反応させると、赤色キノン色素を生成する。この色素をコリンエステラーゼＢ－テストワコー（和光純薬製）を用いて、分光光度計で測定することにより、上記ＰＬＤの活性を測定した。１単位（Ｕ）の酵素活性の定義を、１分間に１μモル（mol)のコリンを遊離することとする。

【００７０】まず、反応基質混合溶液を調製する。上記反応基質混合溶液におけるチューブ１本当たりの混合組成を、H_2O 40μＬ、200 mMのTris-HCl(pH=7.4) 20 μＬ、10mM の$CaCl_2$ 10μＬ、１％Triton X-100 10 μＬ、50 mg/mLのLecithin Emulsion 10μＬとした。上記Lecithin Emulsion は、卵から得られたLecithin 500 mgを1 mL のジエチルエーテルに溶解し、水で 10 mLにメスアップしたものを超音波懸濁させたものである。

【００７１】ＰＬＤの活性測定では、ＰＬＤ酵素溶液 10 μＬ に対し、上述の反応基質混合溶液 90 μＬ を混合し、よく攪拌し、37℃で10分間反応させた。反応後、0.5 mMのEDTAを加え、反応を停止させた。次に、基質酵素剤（コリンオキシダーゼ、ペルオキシダーゼ、フェノール）500 μＬ を加え、37℃で５分間保温し、反応停止液500 μＬ を加え、反応を停止させた。

【００７２】次に、検量線を作成する。水と、コリンエステラーゼＢ－テストワコーの基準液とを、それぞれ、150 μＬ と0 μＬ、140 μＬ と10μＬ、130 μＬ と20μＬ、120 μＬ と30μＬ で混合し、それらの吸光度（波長 505 nm ）を分光光度計にてそれぞれ測定した。それぞれの吸光度の値が、0 U/L 、500 U/L 、1000 U/L、1500 U/LとなるＰＬＤ活性に相当するとして検量線を作成した。この検量線を用いて、試料中のＰＬＤ活性を決定した。

【００７３】（８）電気泳動法（SDS-PAGE)

ＰＬＤの分泌発現を確認するため、12.5％の分離ゲルを用いて、SDS-PAGE電気泳動を行った。電気泳動においては、培地サンプル 20 μＬ を用いた。ゲルの染色は色素(Coomassie Brilliant Blue)を用いて行った。上記電気泳動は、文献（Garfin et al., Methods Enzymol. 182, 425-441 (1990) ）に記載の方法に基づいて行った。

【００７４】（９）ＰＬＤの精製

培地中に分泌生産されたＰＬＤの精製は、pH6 における、陽イオン交換樹脂（Macroprep high S (バイオラッド社製など））によって行った。溶出は、塩化ナトリウムの段階溶出（0.5 M)あるいは直線的濃度勾配法（0-0.5 M)にて行った。また、ヘパリンアフィニティークロマトグラフィーも同等の効果を示した。

【００７５】発明者らは、Stv. cinnamoneum（ＩＦＯ 12852) 由来の pld遺伝子のクローニングを行い、各種遺伝子発現系を利用した組換え微生物による、ＰＬＤの高効率生産プロセスを見出した。

【００７６】まず、本発明者らは、以前において、組換えベクターを用いたＰＬＤの発現系として、大腸菌や酵母（Pichia pastoris)を用いた発現系の構築を試みたが、最も発現活性能が高い野性株のStv. cinnamoneumに対し、大腸菌の場合は１／２０、酵母の場合は１／４の発現活性量しか確認することができなかった。ただし、上記の各場合でも、タンパク質としてのＰＬＤの生産量自体は、大量確認できたことから、これらの発現系では、放線菌由来のＰＬＤが天然型の正しい立体構造を形成することが阻害されているため、発現活性量が低くなったものと思料された。

【００７７】山根らも、放線菌（Streptomyces antibioticus)由来のＰＬＤを用いた、大腸菌における分泌生産系の開発を行っているが、最大でも、その発現活性量は約3500 U/L程度であった（Iwasaki et al., J. Ferment. Bioeng., 79, 417-421 (1995) ）。したがって、放線菌ＰＬＤの高分泌生産系の開発には、放線菌の宿主－ベクター系の開発が極めて重要であると考えられた。

【００７８】そこで、本発明者らは、放線菌の一種であるS.lividansを宿主とし、Stv. cinnamoneum由来の pld遺伝子を組み込んだ発現系（ｐＵＣ702-pld 、図３ないし図５に示す）、さらに pld遺伝子に対し、さらにプロモーター領域を組み込んだ発現系（ｐＵＣ702-promoter-pld、図１ないし図３に示す）の構築を行った。構築した各組換えプラスミドを、それぞれ、放線菌であるS.lividansに形質転換し、形質変換した各S.lividansを試験管により培養した。

【００７９】その結果、S.lividans／ｐＵＣ702-pld の発現系においては、野性株のStv. cinnamoneumと同等の発現量しか確認することができなかったが、S.lividans／ｐＵＣ702-promoter-pldの発現系（試験管培養では、20000 U/L ）では、野性株のStv. cinnamoneum（試験管培養では、1300 U/L）に対し、著しく大きな発現量（約１５倍量）を確認することができた。

【００８０】したがって、本発明に係るプロモーターは、所望するＰＬＤ等のタンパク質の菌体外生産において極めて強力であり、ＰＬＤを始めとする種々な組換えタンパク質の発現において極めて有効であると考えられた。

【００８１】そこで、通気攪拌型培養槽を用いて、この発現系S.lividans／ｐＵＣ702-promoter-pldの培養条件の最適化に関する検討を行った。条件としては、前述のTSB培地に初発添加グルコース濃度をそれぞれ、0 g/l 、5 g/l 、15 g/l、30 g/lとし、培養温度28℃、pH=7.0、およびDO（溶存酸素濃度）＝ 2 ppmにコントロールすることで、それぞれ培養を行った。

【００８２】図６に示すように、グルコースを添加しないときは、培地中の分泌ＰＬＤの活性が約 20000 U/Lであったが、図７ないし図９に示すように、グルコースの添加量の増加に従ってＰＬＤ生産量は増加し、グルコースを 30 g/l 添加することにより、培地中に約 30000 U

/LのＰＬＤ活性を分泌生産することができることが判る。このＰＬＤ活性は、野性株のStv．ｃinnamoneumのＰＬＤ活性（同じ TSB培地では 1500 U/L ）と比べると、約２０倍の発現量であり、野性株のStv．ｃinnamoneumのＰＬＤ活性（培地は異なるが最適化し場合では 2000 U/L ）と比べると、約１５倍の発現量である。

【００８３】ただし、初発グルコース濃度を、 30 g/l を超えて大きくしても、菌体量は増加するものの、ＰＬＤの生産量は増加しなかった。これは、過剰なグルコースの存在に起因するカタボライトリプレッションの影響によるものと考えられた。上記カタボライトリプレッションは、異化産物抑制、つまり、グルコースなどの代謝産物によって多数の酵素の合成が阻害されることを意味する。

【００８４】さらに、初発のグルコース濃度 15 g/l の場合について、培地中に分泌生産されたＰＬＤの純度を電気泳動（SDS-PAGE）により、図１０に示すように、確認した。ＣＢＢ色素染色では、夾雑タンパク質がほとんど見られず、ほぼＰＬＤがシングルバンドとして確認することができた。

【００８５】この培地中に分泌生産されたＰＬＤタンパク質を、SDS-PAGEのバンドより定量したところ、約 75 mg/Lであった。この定量値からＰＬＤの活性を計算すると、約 400 U/Lとなった。以前、本発明者らは、Stv．cinnamoneumから精製を行った天然型Ｐ･ＬＤの活性は、 468 U/Lであった(Ogino et wl., J. Biochem., 125, 263-269 (1999))。このことから、本発明において得られたＰＬＤは、天然型のＰＬＤに極めて近い値を有していた。

【００８６】よって、本発明に係るＰＬＤ（タンパク質）の製造方法では、培地中のＰＬＤは、高濃度で、かつ高純度であり、その上、得られたＰＬＤの活性も、天然型のＰＬＤに極めて近いことから、大腸菌を始めとする、従来の各種の発現系での問題点を全て解決できたことが判る。

【００８７】また、本発明に係るＰＬＤの製造方法は、培地中に分泌されるＰＬＤ純度が高いことから、ＰＬＤの分離回収が、菌体除去後に、一段のイオン交換カラムを用いたイオン交換クロマトグラフィーで可能であるので、極めて、迅速かつ安価に、上記ＰＬＤを精製できて、上記ＰＬＤの生産コストを軽減できるものとなっている。

【００８８】以上のように、本発明者らが見出した放線菌由来のプロモーターをベクターに組み込んだ放線菌（組換え微生物）由来のＰＬＤ発現系は、天然型に極めて近い高活性なＰＬＤを培地（つまり菌体外）中に、高純度、かつ高濃度な形態で大量生産できることから、極めて有効なＰＬＤ発現系であることが判る。

【００８９】このように、培地中に、組換えタンパク質を高純度に分泌生産できる発現系は、従来少なく、上記ＰＬＤ以外の、有用な各種のタンパク質生産への応用が想定される。つまり、本発明に係るベクター（プラスミド）の発現カセット（プロモーター－シグナル配列－パリンドローム配列）は、例えば、コレステロールオキシダーゼ等の、各種放線菌由来の有用タンパク質の分泌生産を始めとして、各種有用タンパク質の分泌生産において極めて有効なものと想定された。

【００９０】また、本発明の組換え微生物であるS.lividans／ｐＵＣ702-promoter-pldは、Stv．cinnamoneumと同様に（Fukuda et al., Biotechnol. Lett., 18, 951-956(1996)）、微生物保持粒子（ＢＳＰs)への効率的な固定化を行うことができるため、固定化菌体を利用した、ＰＬＤを始めとする有用タンパク質の連続生産も可能であり、有用タンパク質の生産能を飛躍的に向上させて、上記有用タンパク質の低廉化を図ることができるものである。

【００９１】

【発明の効果】本発明のプロモーターは、以上のように、ストレプトバーティシリウム・シンナモニウムのＤＮＡ由来の塩基配列であり、タンパク質生産のためのタンパク質用塩基配列の上流側に結合されて、上記タンパク質生産能の向上活性を有している構成である。

【００９２】本発明のベクターは、以上のように、上記プロモーターと、上記プロモーターの下流側に、ＰＬＤ等のタンパク質生産のためのタンパク質用塩基配列とを有している構成である。

【００９３】本発明の組換え微生物は、以上のように、上記ベクターが、放線菌等の宿主内に組み込まれている構成である。

【００９４】本発明のタンパク質の製造方法は、以上のように、上記組換え微生物を培養液中にて培養して、タンパク質用塩基配列に基づくタンパク質を生産する方法である。

【００９５】それゆえ、上記構成および方法は、上記プロモーターを用いたことにより、ＰＬＤ等のタンパク質の生産能を向上させることができ、上記タンパク質を工業化により低廉化できて、適用範囲を広げることができるという効果を奏する。

【００９６】

【配列表】

<110> 財団法人新産業創造研究機構　ＴＬＯひょうご
Za idanhou j in Sh insangyosouzoukenkyuk ikou TL0 Hyogo

<120> プロモーター、それを有するベクター、および組換え微生物、並びにタンパク質の製造方法

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<211> 3477
<212> DNA
<213> Streptoverticillium cinnamoneum
<400> 1

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cgtcgggacg tccgtgagcc ccagctcggc ccgcgccgcg tcgaccatgt ccctggcgtg 180
cagcagcccc agttcgccga gcgtgcgacg cagcgcctcc aggtcggcgg ccgtctccgc 240
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ccgcggctcg tccatcgccc ggaactcctc cagcgccccg ccgtaggtgg cccgtgcctc 360
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aatcgttact tcgtttaagg atgcaca ttg ctc cgc cac cgg ctc cgc cgt tta 1434
Leu Leu Arg His Arg Leu Arg Arg Leu
-30

cac cgt ctg acc cgc agt gcg gcg gtc tcg gcc gtc
gtc ctg gcc gcc 1482 His Arg Leu Thr Arg Ser Ala Ala Val Ser Ala Val
Val Leu Ala Ala
-25 -20 -15 -10

ctg ccc gcg gct ccg gcc ttc gcg agc agc ccc tcg ccc gcc ccg cac 1530
Leu Pro Ala Ala Pro Ala Phe Ala Ser Ser Pro Ser Pro Ala Pro His
-5 -1 1 5

ctg gac gcc gtg gag aag gcg ctg cgc gag gtc tca ccg ggg ctg gag 1578
Leu Asp Ala Val Glu Lys Ala Leu Arg Glu Val Ser Pro Gly Leu Glu
10 15 20

ggt gac gtc tgg cag cgc acc gac ggc aac aag ctg gac gcc tcc gcc 1626
Gly Asp Val Trp Gln Arg Thr Asp Gly Asn Lys Leu Asp Ala Ser Ala
25 30 35

gcg gac ccc tcc gac tgg ctg ctg cag acc ccc ggt tgc tgg ggc gac 1674
Ala Asp Pro Ser Asp Trp Leu Leu Gln Thr Pro Gly Cys Trp Gly Asp
40 45 50

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Ala Ala Cys Lys Glu Arg Pro Gly Thr Glu Arg Leu Leu Ala Lys Val
 55                  60                  65                  70
acg gag aac atc tcc aag gcc agg cgc acg gtg gac atc tcc acg ctc    1770
Thr Glu Asn Ile Ser Lys Ala Arg Arg Thr Val Asp Ile Ser Thr Leu
                 75                  80                  85
gcg ccc ttc ccg aac ggt gcg ttc cag gac gcg ata gcc gcc ggc ctc    1818
Ala Pro Phe Pro Asn Gly Ala Phe Gln Asp Ala Ile Ala Ala Gly Leu
             90                  95                 100
aag gcg tcg gtc gcg tcc ggc aac aag ccg aag gtc cgc gtc ctg gtc    1866
Lys Ala Ser Val Ala Ser Gly Asn Lys Pro Lys Val Arg Val Leu Val
        105                 110                 115
ggc gcc gcg ccg gtc tac cac atg aac gta ctg ccc tcg aag tac cgg    1914
Gly Ala Ala Pro Val Tyr His Met Asn Val Leu Pro Ser Lys Tyr Arg
    120                 125                 130
gac gac ctc aag gcc cgg ctc ggc aag gcc gcc gac gac atc acg ctg    1962
Asp Asp Leu Lys Ala Arg Leu Gly Lys Ala Ala Asp Asp Ile Thr Leu
135                 140                 145                 150
aac gtc gcg tcg atg acg acg tcg aag acc agc ttc tcc tgg aac cac    2010
Asn Val Ala Ser Met Thr Thr Ser Lys Thr Ser Phe Ser Trp Asn His
                155                 160                 165
tcc aag ctc ctc gtc gtg gac ggc gag tcg gcc gtc acc ggt ggc atc    2058
Ser Lys Leu Leu Val Val Asp Gly Glu Ser Ala Val Thr Gly Gly Ile
            170                 175                 180
aac agc tgg aag gac gac tac gtc gac acc cag cac ccg gtg acc gac    2106
Asn Ser Trp Lys Asp Asp Tyr Val Asp Thr Gln His Pro Val Thr Asp
        185                 190                 195
gtg gac ctg gcg ctg acc ggc ccc gcc gcg agc tcc gcc ggc cgc tac    2154
Val Asp Leu Ala Leu Thr Gly Pro Ala Ala Ser Ser Ala Gly Arg Tyr
    200                 205                 210
ctg gac acg ctc tgg acg tgg acg tgc cag aac aag agc aac atc gcc    2202
Leu Asp Thr Leu Trp Thr Trp Thr Cys Gln Asn Lys Ser Asn Ile Ala
215                 220                 225                 230
agt gtg tgg ttc gcg gcc tcg ggc ggc gac tgc atg gcc acg atg gag    2250
Ser Val Trp Phe Ala Ala Ser Gly Gly Asp Cys Met Ala Thr Met Glu
                235                 240                 245
aag gac gcg aac ccc agg ccc gcc ggg ccc acg ggc aac gtc ccc gtg    2298
Lys Asp Ala Asn Pro Arg Pro Ala Gly Pro Thr Gly Asn Val Pro Val
            250                 255                 260
atc gcc gtg ggc ggc ctc ggc gtc ggc atc aag gac tcc gac ccc gcc    2346
Ile Ala Val Gly Gly Leu Gly Val Gly Ile Lys Asp Ser Asp Pro Ala
        265                 270                 275
tcg acg ttc cgc ccg cag ctg ccc tcc gcc ccg gac acc aag tgc gtc    2394
Ser Thr Phe Arg Pro Gln Leu Pro Ser Ala Pro Asp Thr Lys Cys Val
    280                 285                 290
gtc ggc ctg ccc gac aag acc aac gcc gac cgt gac tac gac acg gtc    2442
Val Gly Leu Pro Asp Lys Thr Asn Ala Asp Arg Asp Tyr Asp Thr Val
295                 300                 305                 310
aac ccc gag gag agc gcc ctg cgg gcc ctg gtg gcc agc gcc gac cgc    2490
Asn Pro Glu Glu Ser Ala Leu Arg Ala Leu Val Ala Ser Ala Asp Arg
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315 320 325

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Gln Ile Val Ile Ser Gln Gln Asp Leu Asn Ala Thr Cys Pro Pro Ile

330 335 340

gcc cgc tac gac gtc cgc ctc tac gac atc ctc gcc gcc aag atg gcg 2586
Ala Arg Tyr Asp Val Arg Leu Tyr Asp Ile Leu Ala Ala Lys Met Ala

345 350 355

gcc ggg gtg aag gtg cgc atc gtc gtc agc gac ccc gcc aac cgc ggc 2634
Ala Gly Val Lys Val Arg Ile Val Val Ser Asp Pro Ala Asn Arg Gly

360 365 370

gcg gtc ggc agc ggc ggc tac tcg cag atc aag tcc ctg gcc gag atc 2682
Ala Val Gly Ser Gly Gly Tyr Ser Gln Ile Lys Ser Leu Ala Glu Ile

375 380 385 390

agc gac acg ctc cgc aac cgt ctc gcc ctg ctc aag ggc ggc gac cag 2730
Ser Asp Thr Leu Arg Asn Arg Leu Ala Leu Leu Lys Gly Gly Asp Gln

395 400 405

cag aag gcc aag gcg gcc atg tgc tcc acc ctc cag ctg ggg acc ttc 2778
Gln Lys Ala Lys Ala Ala Met Cys Ser Thr Leu Gln Leu Gly Thr Phe

410 415 420

cgc agc tcc gcg agc gcc acg tgg gcc gac ggg cac ccc tac gcc ctg 2826
Arg Ser Ser Ala Ser Ala Thr Trp Ala Asp Gly His Pro Tyr Ala Leu

425 430 435

cac cac aag ctg gtg gcg gtc gac agc tcc gcc ttc tac atc ggc tcc 2874
His His Lys Leu Val Ala Val Asp Ser Ser Ala Phe Tyr Ile Gly Ser

440 445 450

aag aac ctc tac ccc tcg tgg ctg cag gac ttc ggc tac atc gtg gag 2922
Lys Asn Leu Tyr Pro Ser Trp Leu Gln Asp Phe Gly Tyr Ile Val Glu

455 460 465 470

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Ser Pro Glu Ala Ala Lys Gln Leu Glu Ala Lys Leu Leu Asp Pro Glu

475 480 485

tgg aag ttc tcg cag gag acc gcg acg gtc gac cac gcg cgg ggc gtc 3018
Trp Lys Phe Ser Gln Glu Thr Ala Thr Val Asp His Ala Arg Gly Val

490 495 500

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Cys Ser Leu

505

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-1 1 5 10
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Gly Thr Glu Arg Leu Leu Ala Lys Val Thr Glu Asn Ile Ser Lys Ala
65 70 75
Arg Arg Thr Val Asp Ile Ser Thr Leu Ala Pro Phe Pro Asn Gly Ala
80 85 90
Phe Gln Asp Ala Ile Ala Ala Gly Leu Lys Ala Ser Val Ala Ser Gly
95 100 105
Asn Lys Pro Lys Val Arg Val Leu Val Gly Ala Ala Pro Val Tyr His
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Met Asn Val Leu Pro Ser Lys Tyr Arg Asp Asp Leu Lys Ala Arg Leu
130 135 140
Gly Lys Ala Ala Asp Asp Ile Thr Leu Asn Val Ala Ser Met Thr Thr
145 150 155
Ser Lys Thr Ser Phe Ser Trp Asn His Ser Lys Leu Leu Val Val Asp
160 165 170
Gly Glu Ser Ala Val Thr Gly Gly Ile Asn Ser Trp Lys Asp Asp Tyr
175 180 185
Val Asp Thr Gln His Pro Val Thr Asp Val Asp Leu Ala Leu Thr Gly
190 195 200 205
Pro Ala Ala Ser Ser Ala Gly Arg Tyr Leu Asp Thr Leu Trp Thr Trp
210 215 220
Thr Cys Gln Asn Lys Ser Asn Ile Ala Ser Val Trp Phe Ala Ala Ser
225 230 235
Gly Gly Asp Cys Met Ala Thr Met Glu Lys Asp Ala Asn Pro Arg Pro
240 245 250
Ala Gly Pro Thr Gly Asn Val Pro Val Ile Ala Val Gly Gly Leu Gly
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Val Gly Ile Lys Asp Ser Asp Pro Ala Ser Thr Phe Arg Pro Gln Leu
270 275 280 285
Pro Ser Ala Pro Asp Thr Lys Cys Val Val Gly Leu Pro Asp Lys Thr
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Asn Ala Asp Arg Asp Tyr Asp Thr Val Asn Pro Glu Glu Ser Ala Leu
305 310 315
Arg Ala Leu Val Ala Ser Ala Asp Arg Gln Ile Val Ile Ser Gln Gln
320 325 330
Asp Leu Asn Ala Thr Cys Pro Pro Ile Ala Arg Tyr Asp Val Arg Leu
335 340 345

Tyr Asp Ile Leu Ala Ala Lys Met Ala Ala Gly Val Lys Val Arg Ile
350　　355　　360　　365
Val Val Ser Asp Pro Ala Asn Arg Gly Ala Val Gly Ser Gly Gly Tyr
370　　375　　380
Ser Gln Ile Lys Ser Leu Ala Glu Ile Ser Asp Thr Leu Arg Asn Arg
385　　390　　395
Leu Ala Leu Leu Lys Gly Gly Asp Gln Gln Lys Ala Lys Ala Ala Met
400　　405　　410
Cys Ser Thr Leu Gln Leu Gly Thr Phe Arg Ser Ser Ala Ser Ala Thr
415　　420　　425
Trp Ala Asp Gly His Pro Tyr Ala Leu His His Lys Leu Val Ala Val
430　　435　　440　　445
Asp Ser Ser Ala Phe Tyr Ile Gly Ser Lys Asn Leu Tyr Pro Ser Trp
450　　455　　460
Leu Gln Asp Phe Gly Tyr Ile Val Glu Ser Pro Glu Ala Ala Lys Gln
465　　470　　475
Leu Glu Ala Lys Leu Leu Asp Pro Glu Trp Lys Phe Ser Gln Glu Thr
480　　485　　490
Ala Thr Val Asp His Ala Arg Gly Val Cys Ser Leu
495　　500　　505

【図面の簡単な説明】

【図１】本発明のプロモータを含む、ＰＬＤ生産用のＤＮＡの説明図である。

【図２】上記プロモータを含むベクターの説明図である。

【図３】上記ベクターを作成するための他の前駆体の説明図である。

【図４】比較のための、上記プロモーターを省いた、ＰＬＤ生産用のＤＮＡの説明図である。

【図５】上記ＰＬＤ生産用のＤＮＡを含むベクターの説明図である。

【図６】図２に示すベクターを組み込んだ、本発明の組換え微生物による、グルコース無添加における、ＰＬＤの生産能を示すグラフである。

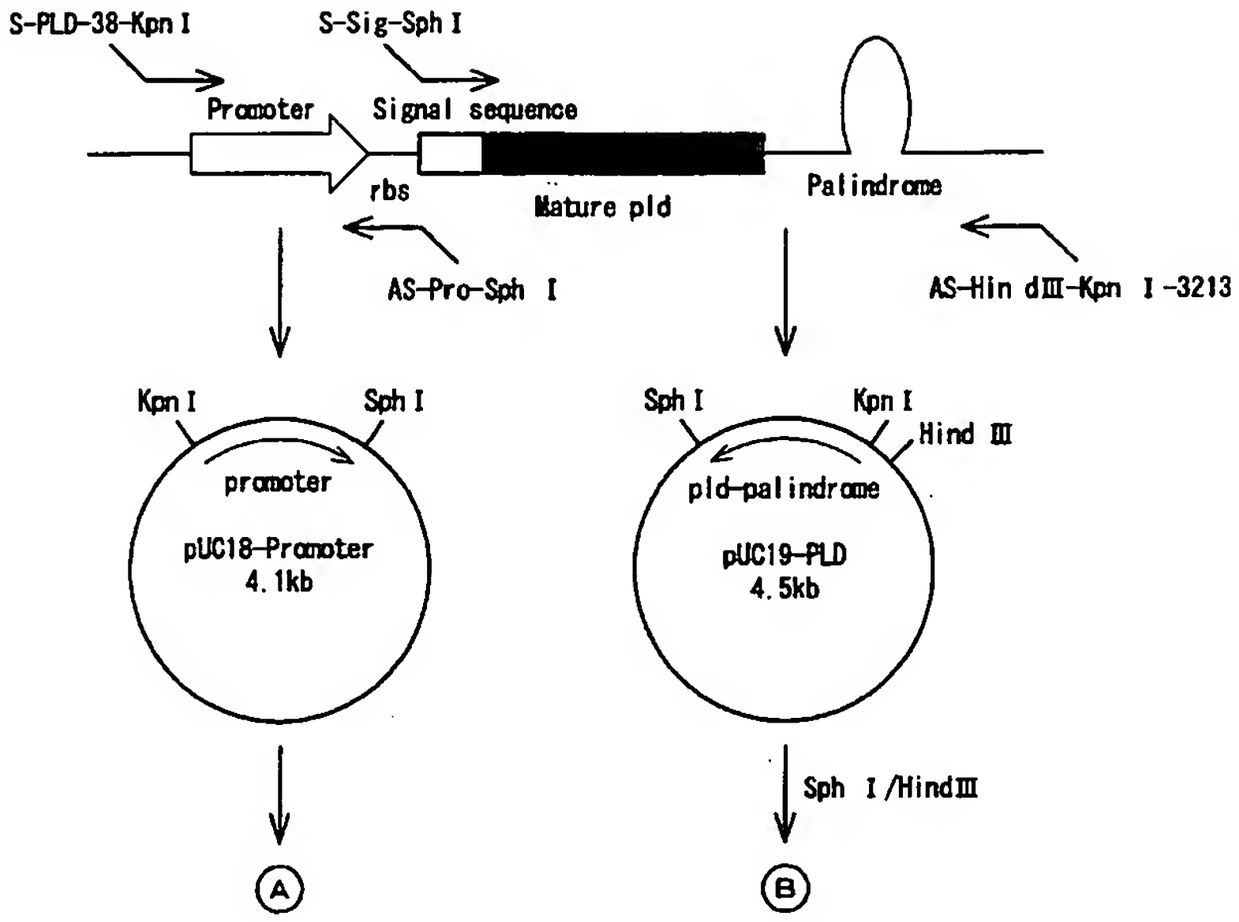
【図７】図２に示すベクターを組み込んだ、本発明の組換え微生物による、グルコース濃度 5 g/Lにおける、ＰＬＤの生産能を示すグラフである。

【図８】図２に示すベクターを組み込んだ、本発明の組換え微生物による、グルコース濃度 15 g/L における、ＰＬＤの生産能を示すグラフである。

【図９】図２に示すベクターを組み込んだ、本発明の組換え微生物による、グルコース濃度 30 g/L における、ＰＬＤの生産能を示すグラフである。

【図１０】図２に示すベクターを組み込んだ、本発明の組換え微生物による、グルコース濃度 15 g/L における培養において、安定期での培養液中のＰＬＤを示すSDS-PAGEの図面代用写真である。

【図１】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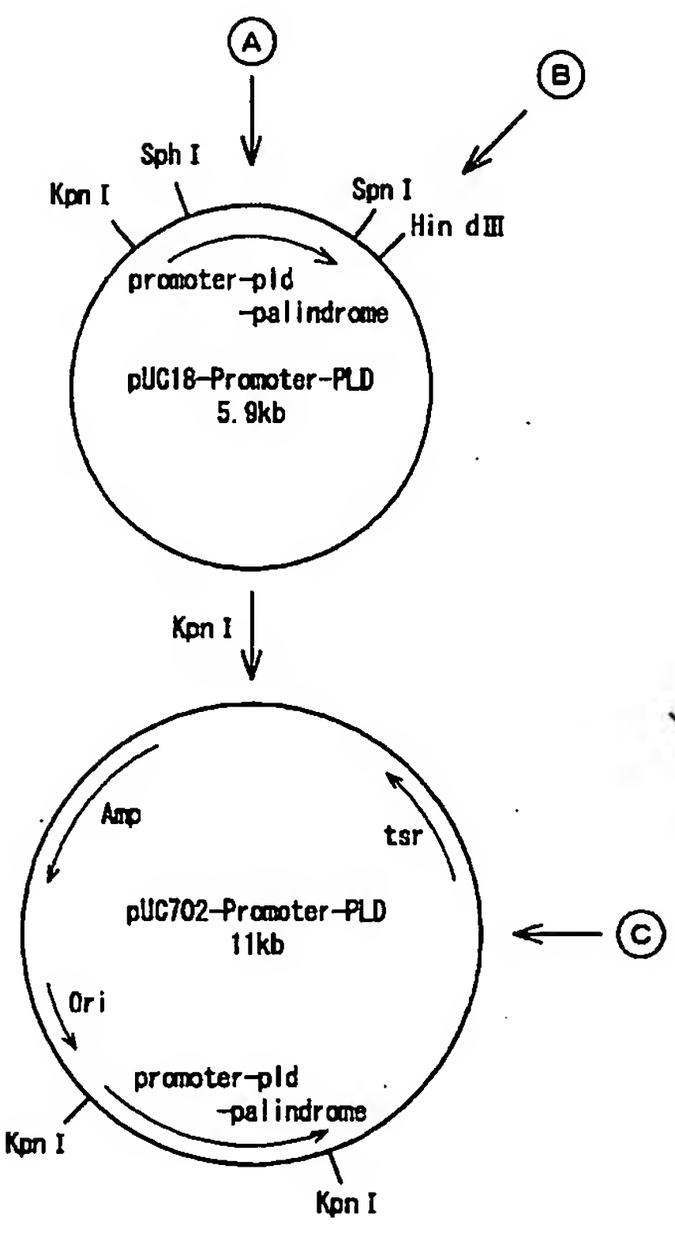
S-Sig-Sph I
Promoter
Signal sequence
rbs
Mature pld
Palindrome
AS-Pro-Sph I
AS-Hin dIII-Kpn I-3213
Kpn I
Sph I
promoter
pUC18-Promoter
4.1kb
Sph I
Kpn I
Hind III
pld-palindrome
pUC19-PLD
4.5kb
Sph I /HindIII
A
B

【図２】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B
Sph I
Kpn I
Spn I
Hin dIII
promoter-pld
-palindrome
pUC18-Promoter-PLD
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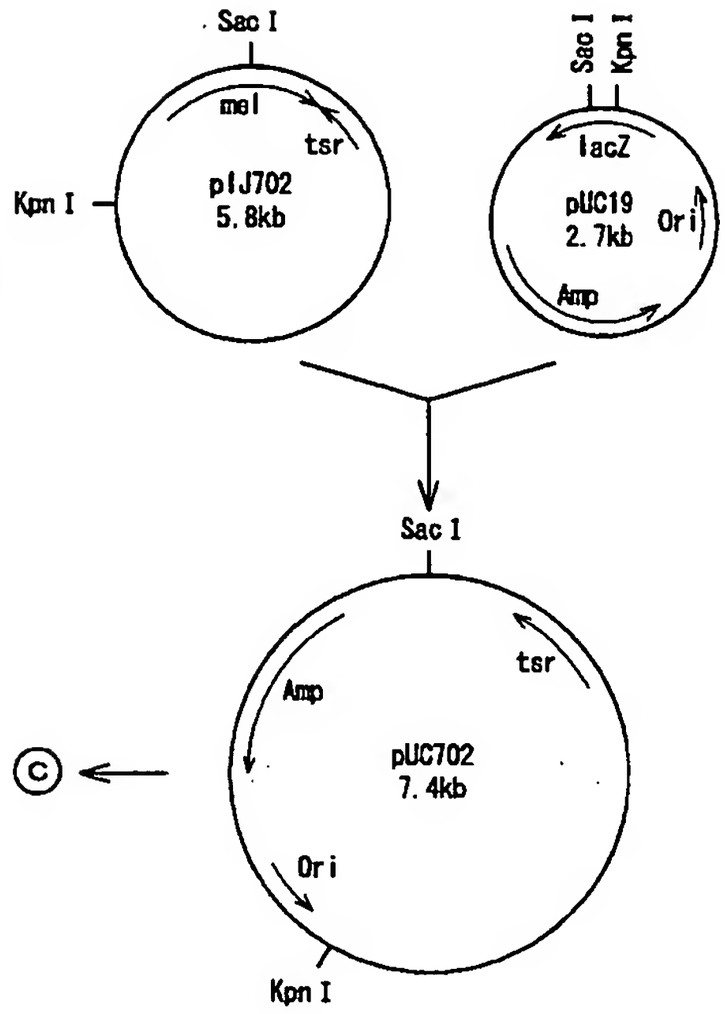
Amp
tsr
pUC702-Promoter-PLD
11kb
Ori
promoter-pld
-palindrome
Kpn I
Kpn I
C

【図３】


Sac I
mel
tsr
pIJ702
5.8kb
Kpn I
Sac I
Kpn I
lacZ
pUC19
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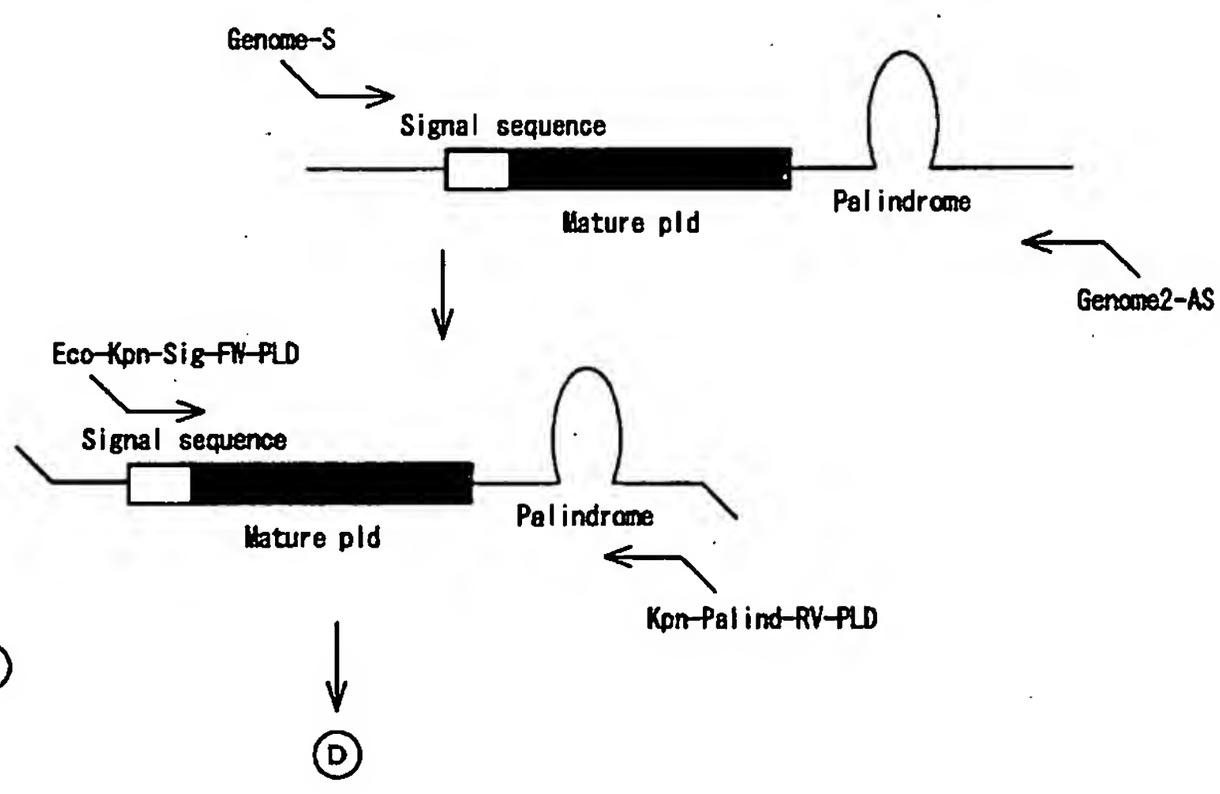
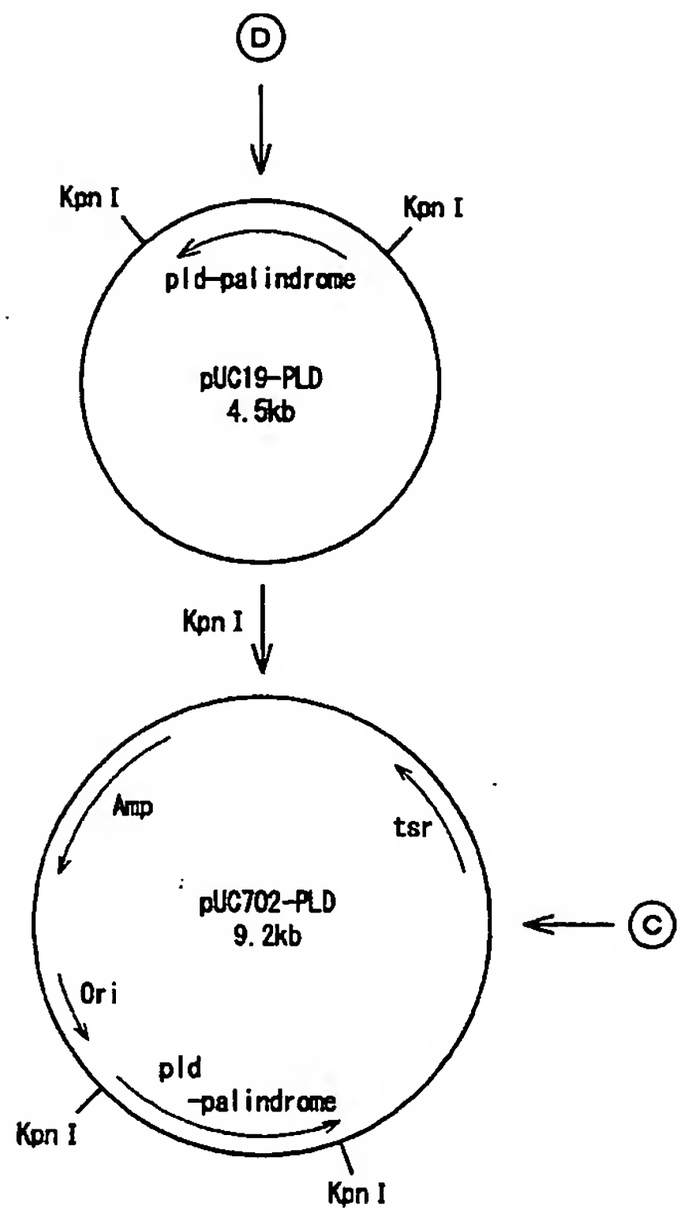
Ori
Amp
Sac I
Amp
tsr
pUC702
7.4kb
Ori
Kpn I
C

【図４】


Genome-S
Signal sequence
Mature pld
Palindrome
Genome2-AS
Eco-Kpn-Sig-FW-PLD
Signal sequence
Mature pld
Palindrome
Kpn-Palind-RV-PLD
D

【図５】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tsr
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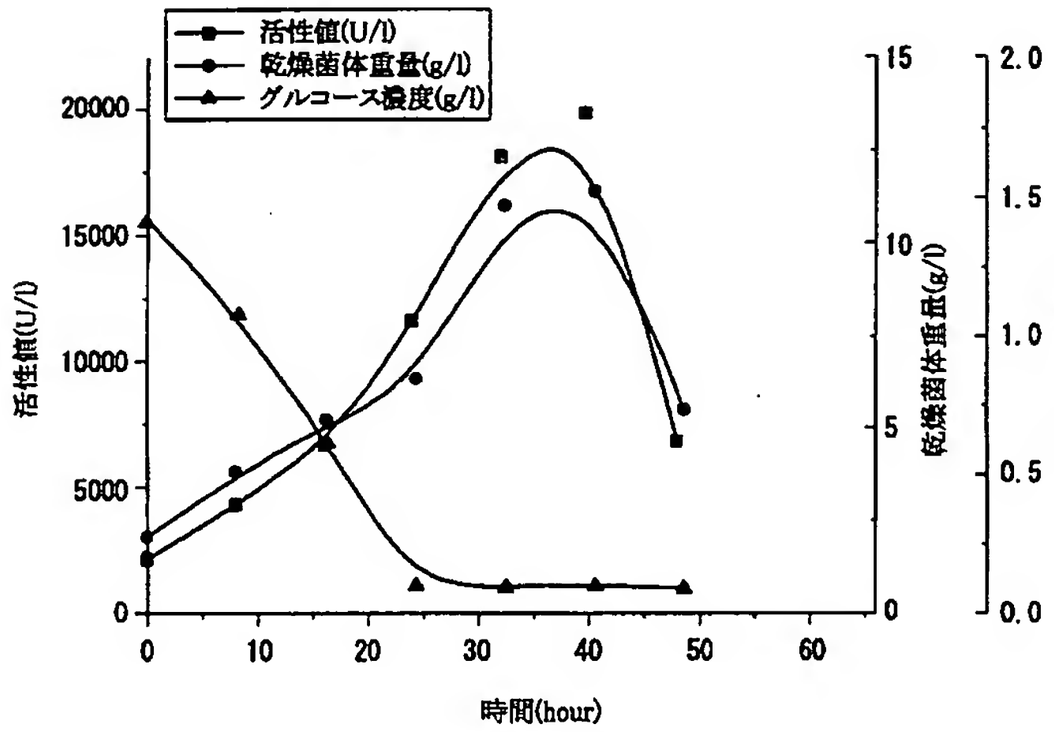
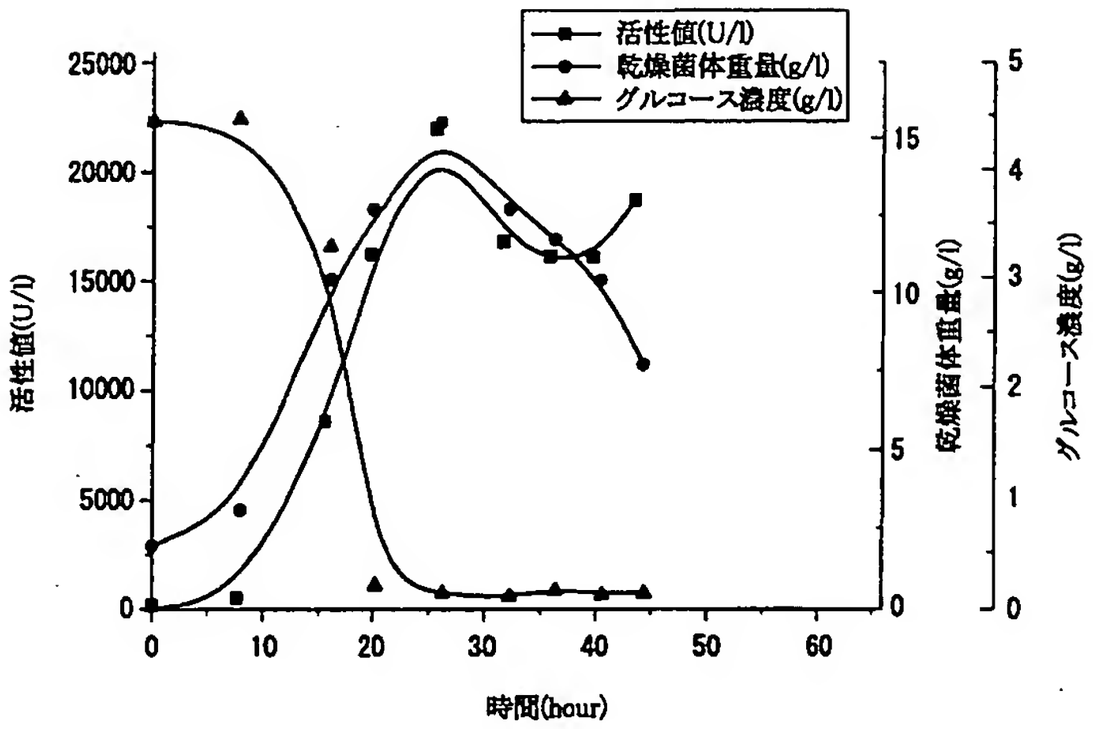
C
Ori
pld
-palindrome
Kpn I
Kpn I

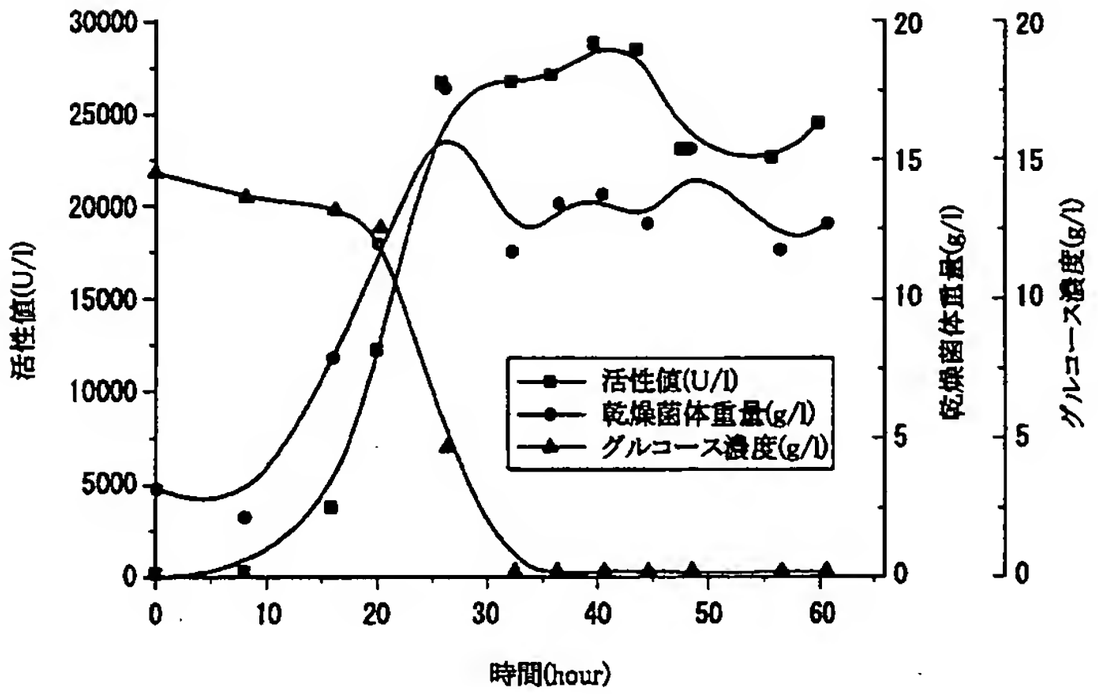
【図６】


活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)
活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)
時間(hour)

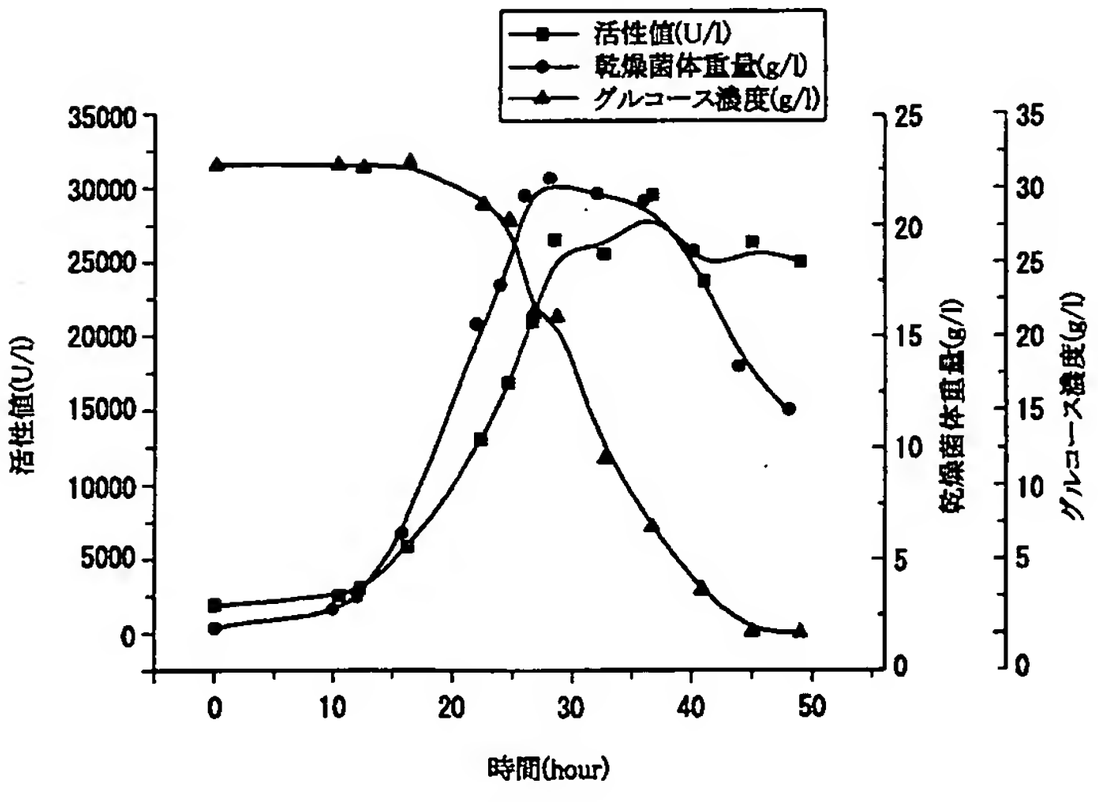
【図７】


活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)
活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)
時間(hour)

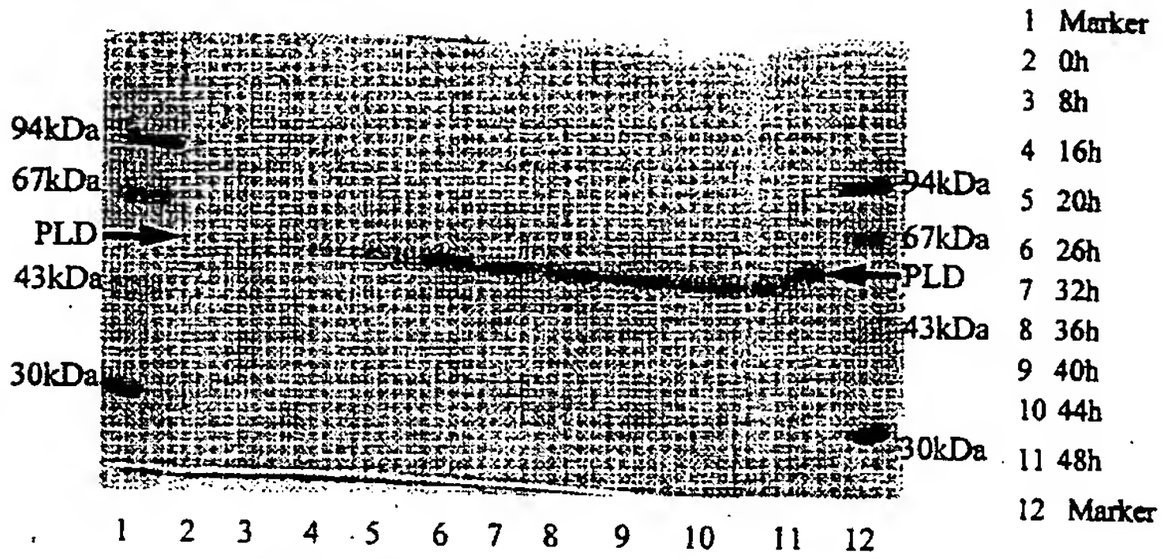
【図８】


活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)
時間(hour)
活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)

【図９】


活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)
時間(hour)
活性値(U/l)
乾燥菌体重量(g/l)
グルコース濃度(g/l)

【図１０】



sample buffer 30 μ l +sample 30 μ lを40/60使用した。

フロントページの続き

(51) Int.Cl.[7]	識別記号	F I	テーマコード(参考)
(C１２N　1/21		(C１２P　21/02	C
C１２R　1:465)		C１２R　1:465)	
(C１２P　21/02		C１２N　15/00	ＺＮＡＡ
C１２R　1:465)		C１２R　1:625)	

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Ｆターム(参考)　4B024 AA20 BA11 CA01 CA04 DA08
EA04 FA02 FA07 FA08 FA15
GA19 GA21 HA01 HA03
4B064 AG01 CA04 CA19 CC09 CC12
CC24 CD09 CE11 CE12
4B065 AA50X AA52Y AB01 AC14
BA02 BA10 BC05 BC08 BD36
CA27 CA60